

nature

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Abracadabra: watch it come down

It is no surprise that 'big science', that is astronomy, space research, particle and nuclear physics, is in for a lean time in the next five years in the United Kingdom. News of shrinking budgets for big science has been coming out for a year or more, generally on occasions that a particular project has "with regret" had to be abandoned. The publication last week of the report for 1974-75 of the Advisory Board for the Research Councils (ABRC) (HMSO, Cmd 6430, 50p) only serves to give the decision a degree of finality (see following article). Nevertheless, since it is the advisory board which is charged with advising the Secretary of State for Education and Science on his responsibilities for civil science and on the allocation of the Science Budget, and since the Secretary of State generally takes that advice, it is appropriate that we should ask, at the time the report appears, how the 'big science' decision was reached, and whether it is in accord with feelings among scientists in general.

Present annual spending in the Science Budget (that is, not by government departments) is £13 million on agricultural research, £29 million on medical research, £19 million on natural environmental research, £9 million on social science research and £96 million to the Science Research Council (SRC). Of this £96 million, £7 million goes on engineering, £24 million on astronomy, space and radio, £35 million on nuclear and high energy physics, £16 million on 'science' (mathematics, physics, chemistry, biology and so on) and the rest on student support and administration. In 1980 the total expenditure is expected to be at exactly the same level in real terms, but the SRC's operations will have shrunk nearly 10% while all the other council's operations will have grown by between 6 and 10%. Within the SRC, the nuclear physics board will have had to cut back its expenditure by roughly 25%, astronomy, space and radio by roughly 16%—others will be slightly up.

The figures are actually bleaker still for big science. There has been some criticism of the growth permitted to all other activities as Buggins' turn, but the ABRC is possibly right in its assessment that increased experimental sophistication and a preponderance of young researchers probably require such financial growth just to maintain existing capabilities. Thus 25% off nuclear physics is probably more than 30% off capabilities. Finally, two major subscriptions, to CERN and the European Space Agency, account for 40% of the present expenditure on big science. These may not be renegotiable; the sum total of all this could be nearly

a halving of the SRC's discretionary activities in big science by 1980.

Why is big science to be cut so severely to maintain the *status quo* elsewhere? The ABRC concedes that in these scientific fields the UK has a high reputation, and it admits that prospects of advance are "particularly good". "On the other hand, the big sciences engage relatively small numbers of research workers . . ." This is an argument with strange echoes from the most recent Commons Select Committee report—too much is in the hands of too few. It is a depressingly bad one for the ABRC to be putting forward, suggesting that, for all the agonising, it is size that has been the criterion, not quality nor even (and this would have been easier to swallow) lack of obvious pay-offs to the taxpaying public.

The ABRC was nearly unanimous in its decision. What is surprising is that the composition of the ABRC should allow it to be such. Big science expenditure is a third of all the science budget yet amongst all the research council heads, chief scientists of government departments and civil servants and academics who served on the board when the decisions were being made, only one out of nineteen had had any first hand experience of big science. Even more surprising, although the board offers seats to departmental chief scientists whose contributions to the support of non-departmental research projects can be as small as a couple of million pounds, it cannot include the Chief Scientist, Ministry of Defence, whose ministry is spending £550 million this year on research and development, but which does not sponsor non-departmental research projects by the Rothschild method. Quite apart from the experiences of the present incumbent, Sir Hermann Bondi, which would have been particularly relevant to recent discussions, it does seem wrong that a ministry which recruits large numbers of graduates and PhDs, which runs the Meteorological Office, and which co-operates in all manner of ways with outside organisations should have no formal say in the shaping of the future of Britain's civil science.

It would obviously be foolish to expect a slavishly democratic ABRC in which every interest was represented proportionally; it is clearly much better that reliance should be placed on a body of people informed by non-partisan commonsense. But there are aspects of the decision on big science and the poorly articulated reasons behind it which should at least make us wonder whether we have yet got the ABRC exactly right.



Research on the rack?

Chris Sherwell looks at two government documents which do not bode well for Britain's research effort

LAST WEEK, half-way between the publication three weeks previously of the Labour government's White Paper on Public Expenditure and its forthcoming revelations in the Budget, most of the country was being suitably distracted from the immediacy of its economic troubles by the resignation of Mr Harold Wilson, the Liberal Party's leadership problems and the state of Princess Margaret's marriage. Not so the scientific community. Two documents came out which directly affected its well-being. Neither can be said to contain any unexpected shocks, but the implications are that the fate of British scientific research may be hanging in the balance now more than ever: certainly the so-called "party is over" mentality has arrived.

The first document was the report of the Advisory Board for the Research Councils (ABRC)*. The report, the ABRC's second, covers the two years of its operation from the beginning of 1974 to the end of 1975; the first, published in June 1974, covered the year of its work following its formation in November 1972.

The ABRC's focus, understandably, is finance, for one of its main tasks is to advise (and its advice is usually accepted) the Minister of Education and Science, within whose departmental ambit it falls, on the allocation of the department's Science Budget. The allocation is principally amongst the Research Councils—that is, the Science Research Council (SRC), the Natural Environment Research Council (NERC), the Medical Research Council (MRC), the Agriculture Research Council (ARC) and the Social Science Research Council (SSRC)—

the rest of the budget, amounting only to about 3%, is accounted for by the British Museum (Natural History) (NHM) and the Royal Society (RS).

It is through the Research Councils, which together with the University Grants Committee (UGC) constitute the "dual support system", that the government fulfils its strategic aim of maintaining a fundamental capacity for research in Britain. The Science Budget itself has grown in financial and in real terms over the last three financial years, and will do so again in the coming year (see Table 1). But for the SRC, whose share of that budget has increased up to a high point this year of more than 56%, a fall in that share is now indicated for the coming year, to 54.2%, and the expectation is for a fall to about 52% by 1980-81. More significantly, over the last two and the coming financial years, the SRC has experienced and will experience an absolute decline in real terms in the resources it receives. Annual percentage growth of 3.2% in 1973-74 gives way to negative real growth in succeeding years of 2.2%, 2.2% and, in 1976-77, 1.6%. It is a measure of the change that, in the seven years before 1973-74, growth was less than 3.2% only in 1969-70.

That change, moreover, is not regarded as a temporary phenomenon. The ABRC's guidelines regarding average annual percentage growth (also shown in Table 1) includes for the SRC a rate of -1.9% until 1980-81; and, for this year at least, the gap between the guidelines for the SRC and the other Councils will widen. As for the Science Budget as a whole, this is expected to remain at its present level up to 1980 apart from the 1.7% increase for the coming year, thus confirming both the gloomy prog-



ABRC Chairman Sir Frederick Stewart

nosis of the White Paper on Public Expenditure and the previously indicated intentions of aiming for nil-growth.

The "keynote" of the ABRC's policy over the coming years, as its second report points out, was "re-deployment away from big science (high energy nuclear physics and astronomy, space and radio sciences) to protect prospects for other natural sciences and social science" (see Figure 1). The consequence for the distribution of the SRC's resources within its own constituent parts is that nuclear physics and ASR (astronomy, space and radio) are the ones to suffer (see Figure 2). Thus, following the SRC's 1974 decision that it was unable to finance a major new £20-millions radio telescope at Jodrell Bank, and its 1975 decision (in light of the German decision approving Germany's PETRA accelerator project) that international finance would not be forthcoming for its proposed EPIC accelerator, it now looks as though existing big projects are in jeopardy. The most important is NIMROD, the 13-year-old 7000 MeV proton synchrotron at the SRC's Rutherford Laboratory, and a final decision is expected soon. A decision has already been taken regarding the closure of the NINA Synchrotron Radiation Facility.

*Second Report of the Advisory Board for the Research Councils (HMSO, Cmnd. 6430).

Table 1 Distribution and annual growth of the Science Budget

	1973-74			1974-75			1975-76			1976-77			Average annual % growth guidelines to 1980-81
	£ million	% of total	Real annual % growth	£ million	% of total	Real annual % growth	£ million	% of total	Real annual % growth	£ million	% of total	Real annual % growth	
ARC	16.824	12.0	4.0	15.114	10.2	-3.7	13.171	7.7	-0.6	18.33	8.5	3.3	1.8
MRC	25.664	18.3	2.7	26.144	17.2	-1.7	29.022	16.9	-1.0	37.36	17.3	2.7	1.7
NERC	15.796	11.2	4.4	16.066 ¹	10.5	-3.0	19.252 ¹	11.2	0.2	26.05 ¹	12.1	2.3	2.0
SRC	71.429	50.9	3.2	83.665 ¹	55.1	-2.2	96.713 ¹	56.4	-2.2	117.19 ¹	54.3	-1.6	-1.9
SSRC	5.854	4.2	8.6	6.767	4.4	5.7	8.749	5.1	6.8	11.18	5.2	2.0	2.0
NHM	2.647 ²	1.9	23.1	2.581 ²	1.7	-2.1	2.873	1.7	1.0	3.86	1.8	—	—
RS	2.170	1.5	3.5	1.499	1.0	2.3	1.733	1.0	—	1.98	0.9	-1.7	1.0
Total	140.384	100.0	3.9	151.665	100.0	-2.1	171.513	100.0	-1.3	215.95	100.1	1.7	

Transferred funds are not included.

¹SRC and NERC figures include an element of the costs of dispersal, not included in the Science Budget.

²Includes cost of buildings borne on Department of Environment Vote (1973-74, £0.9 million; 1974-75, £0.6 million).

Britain's position undermined?

All of which tends to reinforce the view that Britain's position in the "big science league" is likely to be undermined, which some may argue is a bad thing. The ABRC, however, has found itself obliged to take a somewhat different view. Aside from the government's own exhortation that cuts be made, and the fact that these are most readily achieved by axing the biggest and thus most expensive projects, two strands of thought appear to inform the ABRC's overall disposition regarding big science. The first is the general policy one described in its 1974 and 1975 reports: that, apart from the exigencies of overall redeployment, substantial priority also had to be accorded to the SRC's Engineering and Science Boards over the needs of big science, a priority reflected in Figure 2. In 1974-75, fully 60% of the SRC's budget went to big science, while only 11% and 15% respectively went to the Engineering and Science Boards. (Big science's 60% is equivalent to about 33% of the total Science Budget).

It was an imbalance the ABRC thought should be redressed, and one argument its Chairman, Sir Fred Stewart, voiced in justification, apart from the official one of maintaining a "general national scientific capability" overall, was that to support engineering, an activity central to Britain's economic performance, was to improve the chances of more resources for big science later when, with improved economic performance, those resources would be available. No assumptions about such a recovery are being made at the moment, however: the proportion of the total science budget going to big science is projected to fall rapidly from 33% to 27% in 1980-81.

The other important element in ABRC and SRC thinking is inescapable. The SRC faces certain crucial constraints on its capacity to redeploy its funds to the degree it might perhaps wish. These include important international commitments, to the European Space Agency, the European Science Foundation, CERN and so on. These commitments, as they stood last year, amounted to 26% of the SRC's expenditure—and were equivalent to 44% of the Nuclear Physics Board's funds and 24% of the ASR Board's. It is these sorts of commitments, of course, which must be considered in accounting for the sizeable shares going to big science, just as it is the often colossal cost of SRC projects as a whole which must be weighed in any judgments that are made about the majority share of funds which historically goes to the SRC rather than other Research Councils. Both of these factors, by their

nature, must aggravate the impact on British science of the cuts now in store.

Other Research Councils are less harshly treated. The ABRC says it has decided to use extra funds made available by the increase for 1976-77 "in such a way as to reinforce the redeployment policy" it has adopted, bearing in mind that the increase was only a "temporary departure" from the nil-growth trend. It stresses that there is no modification in its policy of distributing resources secured from savings in big science roughly proportionately to the other Research Councils and to the Engineering and Science Boards of the SRC. As for maintaining the basic research "floor", which it endeavours to do through an improving co-ordination with the UGC, the ABRC says there is "likely to be a need for greater selectivity and concentration of resources on the part of the Research Councils".

White Paper on Defence

The trouble is, British scientific research is not starting to go through a particularly lean period just because of the reductions in funds available to big science, to the SRC, or even to the less badly hit ARC, MRC and NERC. A second document released last week, also in the wake of the Public Expenditure White Paper, is rather more difficult to interpret, but it tends to reinforce the pessimistic view. That document was the government's annual White Paper on Defence†. It is worth remembering, if only for the sake of perspective, that the Department of Education and Science's Science Budget in 1975-76, for instance, accounted for only about 12½% of the total government expenditure on research and development—a level that has in fact tended to fall in recent years (it was closer to 17½% in 1971-72, for example). In 1975-76, when the Science Budget was around the £150 millions mark, government research and development spending in what is classified as "higher and further education" was approximately £116 millions, in "trade, industry and employment" it was about £246 millions—and in defence it was over £550 millions.

Not all of the figure ascribed to defence, of course, goes to pure research. A research and development figure of £702 millions in the latest White Paper compares with last year's forecast (at the same price level) of £669 millions and represents 12% of the overall defence budget of some £5,600 millions. Of this £702 millions, £107 millions is forecast expenditure on research, while the rest goes to

†Statement on the Defence Estimates 1976 (HMSO, Cmnd. 6432).

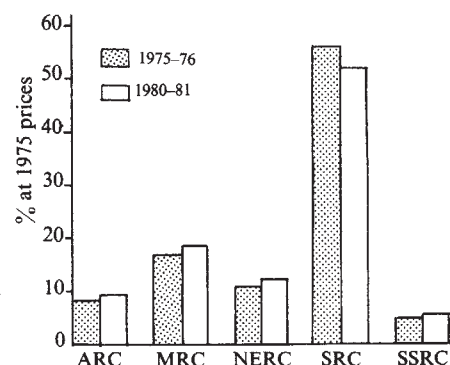


Fig. 1 Research Councils' percentage shares of the Science Budget 1975-76 and 1980-81, as reflected in 1975 guidelines.

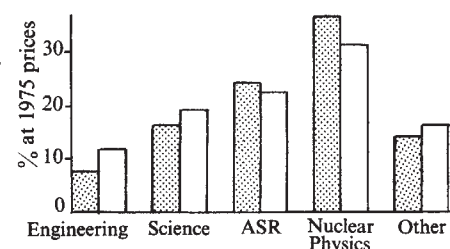


Fig. 2 SRC Boards' percentage shares of the SRC Budget 1975-76 and 1980-81, as reflected in SRC 1975 Forward Look.

"development of approved systems"; of the £107 millions, about two-thirds, the White Paper says, relates to work, including exploratory development, in direct support of projects in the equipment programme. The remaining one third relates to longer term research. Another £22 millions is to be spent on research work "directed to civil objectives".

The breakdown of the £702 millions is not very revealing: £258 millions is attached to military aircraft, £74 millions to guided weapons, £97 millions to "other electronics", £73 millions to ship construction and underwater warfare, £58 millions to "ordnance and other army", £139 millions to "other research and development"—and £3 millions to meteorological research and development. What is revealing, though, is the degree to which the overall cutbacks in defence are forcing the closure of research establishments attached to the ministry. Economies in the Ministry of Defence's research programme, which is organised into 18 major fields (aerodynamics, electronics, armaments and so on) and is done in defence-related industries and university research laboratories as well as the ministry's own establishments, have forced the ministry not only to decide on the second stage of its rationalisation of its research and development programme but also to proceed with "and where practicable to accelerate" it.

As a result, a number of sites will be closed "in due course", some "as soon as possible" but most within five to seven years. They include the Chemical Defence Establishment outstation at Nancekuke in Cornwall, reportedly associated with the development of nerve gas and CS riot gas; future levels of research and development on "defence against chemical and biological warfare", carried out at the parent Chemical Defence Establishment and the Microbiological Research Establishment (both at Porton), are to be reviewed "with the object of making significant economies". They also include the Admiralty's engineering, oil and materials laboratories, and one site of another of the Admiralty's laboratories, as well as a site of the Explosives Research and Development Establishment. In addition, there will be "a further cut on extramural research with industry and the universities where for the time being we shall have to sacrifice some of the longer-term work". Concentration of activities in the field of explosives will enable the work of closed sites to be carried out at the Atomic Weapons Research Establishment and the Royal Armament Research and Development Establishment. Other work will be concentrated as a result of the naval laboratory closures. Redundancies are inevitable, and could amount to many hundreds.

The underlying aim of the rationalisation is to complete "the framework of a research and development organisation based on four main systems establishments—sea, land, air, and underwater—complemented and supported by a number of technology establishments". The aim, in other words, is similar to the comparable one in the civilian area which the Rothschild White Paper foreshadowed, namely, better and more efficient co-ordination of government-backed scientific research and development. While the cuts in defence appear to be encouraging the pace of such a ration-

alisation, though, the redeployment policy of the ABRC as implemented through the SRC is looking increasingly like an issue separable from the other main focus of the ABRC report, the more universal application of the "customer-contractor principle".

"Customer-contractor principle"

Government departments which act as regular "customers" for Councils to which they "contract" research are represented on both the relevant Councils themselves and the ABRC through the comparatively recent institutions of their own Chief Scientific Advisers (CSA). The "customer-contractor principle", outlined by Rothschild in respect of research and development conducted in accordance with objectives formulated by the departments themselves, has already been in operation for some time within the Ministry of Defence through its CSA; its contractor is the Procurement Executive. The principle should have been implemented within three years (that is, by next week) by certain other departments: thus, some of the funds previously lying with the ARC, MRC and the NERC (contractors) should by now have been transferred to their respective customer departments for the departments' own use, which could perhaps be outside the Councils through private research establishments.

The ABRC report is not discouraging about progress in this respect, at least regarding the ARC and MRC. But it acknowledges the complexity of the case of the NERC and its customers, which stems from the sheer diversity of its activity: "There will continue to be some difficulties", the report says, "to which the Council and probably the Board itself will need to give careful thought". But while the ABRC recognises these matters, it studiously avoids the related question of co-ordination amongst the CSAs themselves. That co-ordination, such as it is, is currently pursued largely through the ABRC itself, for at the

moment there is no CSA for the government as a whole: the last one, Sir Allan Cottrell, has not been replaced, and the presence on the ABRC of Sir Keith Berrill, head of the Central Policy Review Staff which advises the Cabinet on strategy, was affirmed as being in his personal capacity only.

These issues are not likely to disappear. But for the moment the main questions surround the government's financing of British scientific research. The country's research effort, while not a party issue, is a political issue insofar as it has to be resolved at the political level. At the moment, however, it manifests itself to the scientific community only as a matter to be rather mechanically resolved at Research Council and ABRC level, and is largely a question of slicing up a financial pie whose size is fixed by Treasury *diktat*. Not only is the size of the pie itself not a major object of contention; the manner in which it is sliced was, as Sir Fred Stewart vigorously insisted last week, the product of consensus. The degree to which the claims of participants in the various branches of scientific research are recognised is unclear, but, in the case of the budget handled by the SRC, it is not obvious that its size, both absolutely and in relation to the other Councils, is an issue thoroughly discussed any lower than the SRC itself.

Thus, whether or not it is true, as the House of Commons Select Committee on Science and Technology recently argued, that SRC funds are directed largely at the behest of a small charmed circle of scientists, it seems clear that that alleged circle may now be losing (if indeed it is conducting) any fight to preserve the share of total funds it has quietly thought itself entitled to in the past. The difficulty, of course, is that in a new nil-growth era, when even the country's defence effort is under scrutiny, Britain's scientists as a whole, like the rest of the country, will over the next few years be losing as well. □

USSR

Broom at the top

Following the disasters of Soviet agriculture during the last Five-year Plan, the USSR Minister of Agriculture Dmitrii Polyanskii has been relieved of his post. Polyanskii was not re-elected to the Politburo at the end of the recent Twenty-Fifth Party Congress, when his Ministry faced sharp criticism, notably from Aleksandr Lyashko, the Prime Minister of the Ukrainian SSR; he did, however, retain his seat on the Central Committee of the Party. Now his dismissal as scape-goat for his

Ministry appears to be a tacit acknowledgement that the criticisms were well-founded, and that more than "abnormal weather conditions" were responsible for the agricultural short-fall.

The new Minister of Agriculture, Valentin K. Mesyats, is a graduate of the Moscow Agriculture Institute. From 1965–1971, he was Minister of Agriculture of the Russian SFSR. Since then he has held the post of Second Secretary of the Communist Party of Kazakhstan (one of the Republics most involved in the Virgin Lands scheme). He is a Member of

the Central Committee, but not a member of the Politburo.

Two newcomers to the Politburo fill the places left by the departure of Polyanskii and the 80-year-old Anastas Mikoyan. They include Dmitrii Ustinov, who has for some 10 years been closely connected with the Soviet's armaments programme, including nuclear arms and missiles. This appointment gives two seats on the 16-man Politburo to military interests—the other being held by Minister of Defence Marshal Andrei Grechko.

Vera Rich

USA

Siting the sun set

In the United States, the Energy Research and Development Administration (ERDA) has faced numerous complications over its solar energy plans. And, as Colin Norman reports from Washington, problems remain

IT HAS been several years since a major political scrap broke out in the United States over the location of a major new research facility, for the good reason that there haven't been many new facilities established recently. But in the next few months every state is expected to compete for a scientific prize—a Solar Energy Research Institute, to be established by the Energy Research and Development Administration (ERDA).

The location of the institute won't be the only source of dispute, however, for last week ERDA announced plans for the facility which suggest that it will be a much more modest enterprise than many of its proponents had envisaged.

Plans for the institute have been through a long, and difficult, gestation period. Approved by Congress as part of a bill passed in November 1974, the institute, known by its acronym SERI, was expected to be a major national laboratory for planning and carrying out government research on solar energy. Congress left ERDA to decide exactly what the role and scope of SERI should be, and it also asked the National Academy of Sciences to assist ERDA in the planning.

When the bill was approved, it was generally assumed by SERI's congressional sponsors that the institute would rapidly evolve into the focal point for the government's solar energy programme, and that it would develop into a major facility along the lines of ERDA's nuclear laboratories such as Oak Ridge or Los Alamos. And that assumption was reinforced by the Academy, which last October recommended that SERI should be provided with a budget of about \$50 million and a staff of 630 by 1980.

Modest effort

ERDA officials also hoped to get moving quickly on a large facility but, following a series of disputes with the White House culminating in the resignation of a senior ERDA administrator, the agency last week announced plans for a much more modest effort. The plans, set out in a formal invitation for public and private institutions to bid for a contract to establish SERI, envisage a modest

beginning over the next three years, with no absolute guarantee that the institute will ever develop into ERDA's leading solar energy laboratory.

ERDA, in short, has asked for proposals to establish a solar energy management and research team at an existing institution, with a budget of between \$4 and \$6 million for the first year, rising to \$10–20 million in the third year. In addition, ERDA has asked the bidders to propose a nearby site for a larger, separate facility into which SERI could move at a later date if necessary. The idea is to provide for deliberate, phased growth possibly—though not definitely—to a major national laboratory in the early 1980s.

Under ERDA's direction, SERI will provide analysis and planning for research and development programmes on solar energy, ranging from direct use of sunlight for heating and cooling buildings to electricity generation, wind power, and bioconversion. In addition, SERI will perform some research and development itself. Dr Robert Hirsch, acting assistant ERDA administrator for solar, geothermal and advanced energy, said last week that he expects a contractor and location to be chosen in December, and that SERI will begin operations on January 1, 1977. A decision on whether or not SERI will move to a large, permanent site is expected in two or three years, he added.

Cautious approach

ERDA arrived at that pared down proposal after the White House Office of Management and Budget (OMB) had vetoed its earlier plans for a more ambitious facility. OMB, understandably, argued that ERDA should proceed with caution, moving to a large facility only if the need for a major enterprise is demonstrated. OMB's reluctance to move with dispatch on SERI, and on other parts of the solar energy programme, led to the resignation in January of John M. Teem, Hirsch's predecessor in charge of ERDA's solar energy efforts.

The cautious approach embodied in the proposals is likely to provide considerable ammunition for solar energy proponents, who have long argued that the solar programme has been held back in the past few years while funding for nuclear energy has been growing by leaps and bounds. Though government expenditures on solar energy and development have grown from \$200,000 in 1972 to an anticipated \$116 million next year, the total

proposed for the nuclear programme next year amounts to nearly \$3,000 million. Solar energy proponents are particularly fond of pointing out that the breeder reactor alone—which, like some uses of solar energy isn't expected to make any contribution to energy supplies until the 1990s—amounts to \$880 million next year.

Nevertheless, since SERI is likely to be a prestigious institute which will attract top-level talent and perhaps some associated industry, there is likely to be fierce competition between the states for the contract. The competition in fact started several months ago when several states set up committees to plan their strategies for securing SERI. ERDA has also been inundated with inquiries from interested institutions.

The last tussle over the location of a major research facility in the United States was during the Johnson Administration, when the National Accelerator Laboratory was up for grabs. In the end, that facility was located in Illinois because, according to widespread speculation, Johnson needed the support of Illinois Senator Everett Dirksen, then Senate minority leader, on several key items of legislation. ERDA, however, is anxious to avoid any suspicion that such squalid political deals will decide the site for SERI, and it has insisted that the location will be chosen on strictly scientific and other "factual" criteria.

ERDA taken aback

ERDA officials were therefore somewhat taken aback when President Ford told a Colorado energy symposium last September that Arizona, New Mexico and Florida are the leading contenders to house SERI. Ford, moreover, told officials from Florida's Brevard County recently that their region would receive "excellent consideration" as the site for the institute.

Be that as it may, Hirsch said last week that SERI could be located anywhere in the United States, and that he expects to receive proposals from all 50 states. He noted that the institute will be performing a range of work on solar energy and that some technologies (generation of electricity from wind, for example) don't need large quantities of sunlight. It should not therefore be assumed that SERI must be located in the sunny southwest, he said.

Proposals must be submitted to ERDA by July 15, a site selection board composed of ERDA officials will evaluate them, and a final decision will be taken by ERDA administrator Robert C. Seamans, Jun. in December. □

CANADA

Eldorado radiates Hope

David Spurgeon reports from Ottawa on the Port Hope affair

As a result of continuing public concern about radioactive contamination in the Port Hope area of Ontario, a federal-provincial task force drawn from different departments of the national and Ontario governments was formed late last month to supervise a clean-up of the area. The force will also help the Atomic Energy Control Board (AECB) to discuss the significance of radioactivity in other locations in Canada.

The formation of the task force was announced by the Federal Minister of Energy, Mines and Resources, Alastair Gillespie, after documents were tabled in the House of Commons reporting on the Port Hope situation. These revealed a total of 109 locations in 25 areas of Canada where radioactivity is known or suspected.

Radioactivity from radon gas was discovered last year in a Port Hope school and at several other sites, including private homes. It came from waste disposal from Eldorado Nuclear Limited, which had recovered radium

in the 1930s and 1940s from pitchblende-radium-silver ores mined in the Northwest Territories. The abundant pitchblende (uranium) content was largely discarded at that time because it was thought to be of little value. The radium extracted was used for cancer treatment and in luminous dial painting.

During that period, wastes were disposed of at Port Hope in piles on and close to the Eldorado plant. Toxicity and radiation hazards standards were not then well established. Some of the waste is believed later to have found its way to various parts of the town in the form of fill that was trucked about.

Wastes were also sold to a company in the United States for recovery of metals, which involved shipping the wastes about, and some of the contamination apparently came from spills *en route*, or from storage. A building programme by Eldorado Nuclear Limited may also have resulted in contamination of rubble, fill and reclaimed building materials.

During the Christmas festive period last year, a street-by-street survey of the whole town of Port Hope was

made by the AECB and the Ontario health ministry. Samples of air were collected for analysis when abnormal radioactivity levels were found.

At one private home, air samples taken outside the house showed radon concentrations ranging from 200 to 400 times the acceptable level for continuous exposure. Contaminated fill had been used to fill a ravine behind the house. The health ministry has closed a school, and several families have vacated their homes.

A number of recommendations have already been carried out to remedy the situation in Port Hope. The task force's immediate priority is to complete the investigation and clean-up in Port Hope. The AECB has opened a public information centre there and has assigned two staff officers and two vehicles to the investigation, and plans have been made to carry out an aerial radiometric survey.

If nothing else it seems likely that any future plans to install nuclear capacity on the shores of lake Ontario will be subjected to close scrutiny in the wake of the Port Hope affair. All the same, it remains a source of worry that minor warnings about Port Hope had been given over many years before the latest developments occurred. □

SWEDEN

Generating nuclear heat

The use of nuclear energy, as Wendy Barnaby indicates in this report from Stockholm, is a sensitive issue in Sweden

MR Ralph Nader, the American consumer expert, has said that the Swedes' nuclear energy programme is hindering their ability to work for world peace. At a meeting of environmentalists in Gothenburg, Mr Nader said he was disappointed with the Swedish Prime Minister, Mr Olof Palme, who had in his opinion been too quick to accept arguments for a rapid expansion of nuclear power. (Mr Palme's Social Democratic government plans to have 13 reactors in operation by 1985). If Sweden concentrated on nuclear energy it could not refuse to back other countries making the same choice. But, Mr Nader maintained, the only way to stop the proliferation of nuclear weapons is to stop the spread of nuclear energy for peaceful purposes as well.

Meanwhile, Mr Björn Gillberg—often called "Sweden's Nader"—is waiting for a court decision in one of his

many battles against nuclear power. The case, fought on behalf of a local environment group by Gillberg and a lawyer, Gunnar Michelson, against the State Power Board, concerns permission to operate reactors three and four at the Ringhals plant on the west coast. Technicalities have put the case into the court of appeals of the Water Court, whose jurisdiction is limited to considering whether the reactors could damage their water environment. The only aspect of the plant able to be discussed has therefore been the release of cooling water into the sea.

If permission is given for the reactor to be operated, 175 cubic metres of cooling water per second—the largest release in the world—will flood into the sea through pipes so large that trucks can be driven through them. The environmentalists contend that the effect of a court decision in favour of operation would be to cause a certain number of deaths from the water's radioactivity, and that the court has no right to make decisions which kill people. The State Power Board agrees that every year of opera-

tion would cause some cancers and genetic defects, although its estimation of their number is smaller by a factor of eight than the environmentalists'.

As the reactors are already being built, it is highly unlikely that permission to operate them in some form or another will be refused. Mr Gillberg is of course aware of this, but maintains that nuclear power should be fought on all fronts, no matter how small the odds of winning may be.

Not that Mr Gillberg and his friends should be unhappy with the latest opinion poll on nuclear energy. Fully 54% of the sample wanted no more than the five reactors already built—and this position was taken by 41% of Social Democratic supporters. Only 34% of the sample wanted more than the present five: the rest did not know. More surprising, however, was that 75% of the sample preferred to curb their standard of living in order to avoid an increase in energy use, rather than to raise their standard of living and accept the consequences of using more energy. And the majority comprised 70% of the Social Democratic respondents. The vital question, though, is not the divisions in opinion polls, but the effects they have, in this election year, on public policy. □

IN BRIEF

Marine pollution

Delegates to the Inter-Governmental Maritime Consultative Organisation, meeting in Acapulco this week to discuss marine pollution from ships, will consider UK Government reports which survey national and international counter-pollution arrangements and outline UK contingency plans to tackle major oil spills in territorial waters. While claiming that existing UK measures have already proved partially successful in dispersing a 300-tonne spill in the English Channel last November, the reports stress the need for improved international cooperation. Discussions between France, Norway and Britain are already under way.

Anders move

In a surprise announcement, President Ford last week nominated William A. Anders, Chairman of the Nuclear Regulatory Commission (NRC), to be ambassador to Norway. Anders, a for-

mer astronaut, established a reputation as a tough administrator during the first year of the commission's activities as watchdog over the nuclear industry. Ford indicated that he would nominate Marcus A. Rowden, a former general counsel to the Atomic Energy Commission and a member of the NRC, to succeed Anders.

Cyclamate view

A committee of cancer experts in the USA has failed to reach a definitive conclusion about whether or not the banned artificial sweetener cyclamate is a carcinogen. The committee, appointed by the National Cancer Institute to help the Food and Drug Administration decide whether to lift the ban on cyclamate, noted that most of the tests leading to a significant increase in tumours in cyclamate-treated animals have employed dubious procedures or used strains of animals which have a large incidence of spontaneous tumours, but said the results were a cause for concern.

UK chemical industry

Following last week's National Economic Development Office report urging the UK chemical industry, now at "a decisive point", to reassess existing rates of investment and bring forward planned projects so as to improve international competitiveness beyond 1978, an independent publication from the Chemical Industries Association (CIA) has called on the Government to boost managerial confidence by easing the price and profit controls cutting into available revenue at a time when investment demands are three times higher than in 1974. The CIA report says that the industry's plans to spend £2,800 millions on new plant and equipment over the next three years indicate a willingness to meet projected market demands. The difficulties faced by the industry were simultaneously underlined by the publication of a third report, showing that several leading chemical companies have been trading at a real loss over the past three years.

THE delights of tobacco were recorded in the panegyric "My Lady Nicotine" by James Barric. Perhaps he should instead have used the word "nitrosamine", as the *grande dame* who lurks in the fragrant weed. *N*'-nitrosornicotine (NNN) was identified by Hoffmann and co-workers in unburned tobacco at levels ranging from 2 to 89 parts per million. Most samples contained about 4 p.p.m. This may be compared with about one four-hundredth of this level of nitrosopyrrolidine in bacon, a commodity that is now figuratively as well as literally in the frying-pan for its content of nitrosamines. NNN induces multiple pulmonary adenomas in mice, but just how much of the carcinogenic effect of cigarettes is caused by nitrosamines is not known. The smoke from one cigarette contained 0.14 microgram of NNN, which is non-volatile, and other nitrosamines that are volatile. The well-televized habit of chewing tobacco is familiar to all baseball fans, but I have seen no epidemiological studies on pitchers.

A few weeks ago my lady nicotine (or nitrosamine) came knocking at my door in the guise of a letter from Mr William D. Hobbs, the chairman of the board of the R. J. Reynolds Tobacco Company. I was, he told me, a leader in my community, and, therefore, I would want to know about a technological achievement consisting of a new cigarette, which "draws free and easy" (*sic*). If I were not a smoker, he said, I might wish to refer this

offer of two packages of cigarettes to someone who I thought might be interested. I told him I had a better idea. I believed that all advertising of cigarettes should stop, and that direct

My lady nicotine



THOMAS H. JUKES

mail advertising to start someone in the habit of smoking was particularly pernicious.

My answer to this came from a company executive, Mr Crohn, presumably assigned by Mr Hobbs to respond to anti-cigarette cranks. He sent a letter, and various pieces of literature favouring cigarettes. One

item was an article reprinted from *Executive Health*, a well-advertised publication that sends out appeals for funds to support an Institute for Orthomolecular Medicine. The article was entitled "The Case Against Tobacco Is Not Closed. Why smoking may not be 'dangerous to your health'". Good health, executives! Mr Crohn's letter told me that if advertising of cigarettes were to cease, then "the public would never know that low tar and nicotine cigarettes, which many persons desire, are available in the market place".

The phrase "which many persons desire" rang a faint mnemonic bell of the first casket scene in *The Merchant of Venice*—written before men were persons and coffins were caskets. In this scene, the gold casket has the inscription "Who chooseth me shall gain what many men desire". Upon reading this, the Prince of Morocco says, "Why, that's the lady: all the world desires her". Was Mr Crohn a crypto-Shakespearean? Did he mean many persons desire My Lady Nicotine? But then, in the play, the casket is opened to reveal

"A carrion Death, within whose empty eye

There is a written scroll!"

The scroll, I mused, perhaps said, "Warning: The Surgeon General has determined that cigarette smoking is dangerous to your health".

We shall never know. The warning was written in letters too small to read.

correspondence

Allergic reactions to laboratory animals

SIR—There is a growing concern about allergic reactions in laboratory workers who handle animals but little factual evidence is available. The British Society for Allergy and Clinical Immunology appointed a working party to investigate this subject and here we report preliminary findings, which give some idea of the size of the problem.

We used a questionnaire in two laboratories, one industrial and the other non-industrial, but the results were closely similar, so here we amalgamate them. 474 individuals were questioned. Salient points were, that of this population,

- 23% had had one or more symptoms occurring repeatedly within 12 hours of animal contact
- 9% had had chest, 17% nasal, 10% eye and 11% skin symptoms
- 4% had had to stop working with animals
- after two years of exposure the incidence of allergy did not increase significantly with further exposure
- a family history of allergy was present in 22% of those who reported symptoms, 19% of those who did not. This surprisingly small difference calls into question the usefulness of a pre-employment family-history screen, and is at variance with results from a recent survey in the USA (Lutsky I. I., and Neuman, I., *Annals Allergy*, **31**, 201 (1975)).

We regard the figure of 23% as disturbingly high, higher than those reported recently from the USA (two surveys gave 15% and 11%), and we are starting a much larger survey to investigate factors which may alter the incidence of allergic reactions in exposed populations.

Yours faithfully,

GEOFFREY TAYLOR (CHMN.)
G. E. DAVIES (SEC.)
R. E. C. ALTOUNYAN
H. MORROW BROWN
A. W. FRANKLAND
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Volume publishing

SIR,—I write to draw attention to, to underline, and to generalise from, the exquisite review (March 4, page 82) of the *Handbook of Perception*, which I have not seen. Dr John Mollon lambasts non-editing, a feature not confined to the publishers of the *Handbook*, and unfortunately rife in the field of science publishing.

Progressive specialisation and illiteracy make it clearly more and more difficult for any one author to cover a field in depth: hence the mushrooming of Edited volumes during the last decade or two. A publisher approaches a Name, offers a percentage out of all proportion to the services rendered (which frequently consist in no more than drawing up a list of contributors and acting as a relay station for the receipt of typescripts), and informs the Inland Revenue at the appropriate time.

The contributors are also offered a percentage out of all proportion to the services rendered: I know of a case where the typist was paid more than the author. They are often up-and-coming youngsters who will not heed the warnings of us older hacks. I always point out to younger colleagues that fixed payments are to be avoided like the plague especially if they are made after publication: inflation eats into the terms but the selling price of the book can protect the publisher and editor. I also stress the naïveté of the notion that they will derive any kudos: later references are almost invariably made to the Editor-so-called, with the author's name sunk in Lethe. The crowning insult occurs when a generous publisher sends along 25 reprints, and so ensures a reduction in sales.

It seems to me that there is a case for an agreed code of good conduct to protect the interests of (young) authors who probably cannot afford the luxury of membership of authors' protective associations—which can, in any case, do little more than offer advice. Moreover, if Editors started spelling their designation with a lower-case e, the interests of authors might begin to be guarded, and one of the last relics of child labour expunged from society.

Yours faithfully,

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Alternative refrigerants

SIR—The letter from Birks and Leck, (Correspondence, March 4, page 8) avers that while we can do without aerosol cans, our civilization cannot function without refrigerators. It is then suggested that, if we do not choose to give up refrigerators, we may have to continue tolerating atmospheric pollution by chlorofluorocarbons, even if we ban aerosol cans.

The implication that we are faced with such an awkward choice is not, I think, correct. It is true that we can prevent release of chlorofluorocarbons into the atmosphere by aerosol cans in only one way: ban aerosol cans. It is an essential part of their operation to release the gas they contain.

This is not true of refrigerators, which normally release their refrigerant gas only when junked. This release could be almost completely prevented by

- a law making it an offence carrying a heavy penalty to junk a refrigerator without first having a suitable public or private (licensed) agency remove the refrigerant, or
- much better, requiring a meaningfully large deposit to be left when a refrigerator is purchased and rebating that sum, plus inflation related interest, only upon presentation of proof that the refrigerant has been duly recovered by the designated agency.

We can, in short, keep refrigerators and save our atmosphere, even if it becomes necessary to discontinue using aerosol cans.

Yours faithfully,

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The pace of life

SIR—Hermits can communicate with everyone in their community by standing still (February 19, page 557 and March 18, page 188). But what about pedestrians in downtown Tokyo (population in excess of 10⁷)? Can anyone confirm that they walk at almost 2 m s⁻¹?

Yours faithfully,

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news and views

WHEN a foreign nerve is implanted into a normally innervated skeletal muscle, it grows out over the surface of the muscle fibres but does not synapse with them. If, at this juncture, the normal innervation is removed by cutting or crushing the nerve, then the foreign nerve will now form functional synapses on the denervated fibres. The original nerve eventually regenerates and synapses again with the muscle. This results in muscle fibres which have been 'tricked' into accepting innervation from two sources. The subsequent fate of these dually innervated fibres is currently of great interest to neurobiologists, since one can ask the basic question of whether the foreign innervation is recognised, and somehow suppressed or removed in favour of the original. Such a mechanism could be of considerable importance in trying to account for the great specificity and plasticity of connections that is exhibited by the nervous system.

The principal proponents of such a mechanism have been Mark and his colleagues who have provided evidence that in both goldfish eye muscle (Marotte and Mark, *Brain Res.*, **19**, 41, 53; 1970) and adult salamander muscle (Cass *et al.*, *Nature*, **243**, 201; 1973), the foreign innervation can be functionally suppressed. Furthermore, they have claimed that the suppressed synapses are not retracted, but remain on the muscle fibre and appear normal when examined in the electron microscope (Marotte and Mark, *Brain Res.*, **19**, 53; 1970). The assay for function in the goldfish eye muscle was indirect, involving behavioural testing of the eye movements, and their interpretation of the results is currently controversial since a repetition and extension of these experiments has led to the conclusion that the foreign innervation is not suppressed (Scott, *Science*, **189**, 644; 1975). Meanwhile, in several other situations, including one where the different synapses were formed on a neurone, the dual innervation has proved perfectly stable with no evidence of suppression, even after several months (Frank *et al.*, *Nature*, **247**, 375; 1974 and Purves, *Nature*, **256**, 589; 1975). But in a timely contribution (this issue of *Nature*, page 350), Yip and Dennis have provided clear electrophysiological evidence that suppression does take place in dually innervated fibres of the adult newt. By intracellular recording from individual

Suppression of foreign synapses

from Jeremy Brockes

muscle fibres, they have been able to show that transmitter release from the foreign nerve terminals is reduced as a consequence of re-innervation by the original nerve. Specifically, the amplitude of the synaptic potential decreases during the first two months following re-innervation. The synaptic potential is derived from a number of packets, or quanta, of transmitter that are recruited synchronously by stimulating the nerve. By analysing a number of fibres at different times during the period of suppression, Yip and Dennis have shown that the number of packets recruited (the 'quantal content') declines while the amount of transmitter in a single packet does not change significantly. This is a most important insight into the suppression process, since it characterises it as a presynaptic effect on transmitter release rather than a postsynaptic effect on, for example, the chemosensitivity of the muscle membrane.

An important question, which has yet to be answered, concerns the eventual fate of the suppressed synapses. Are foreign synapses withdrawn from the muscle when the original nerve returns, or do they remain structurally intact but functionally suppressed, as suggested by Mark and his colleagues? When, after six months, the original nerve was interrupted for a second time, Yip and Dennis found that the re-establishment of functional transmission through the foreign nerve appeared to occur more rapidly than after the initial operation. This suggests that the foreign nerve axons remain in relatively intimate contact with the muscle, but does not necessarily mean that foreign synapses are present. In an analogous situation during normal development, it is known that mammalian skeletal muscle fibres are multiply innervated before birth (Redfern, *J. Physiol., Lond.*, **209**, 701; 1970; Bennett and Pettigrew, *J. Physiol., Lond.*, **241**, 515; 1974); when this multiple innervation is

eliminated during development, the extra synapses are completed retracted. Although this process is not identical to that observed by Yip and Dennis, it does illustrate that competitive interactions between synapses can lead to the removal of extra innervation.

Meanwhile, one wonders what rules govern the operation of suppression, and why it did not take place in several other situations. Is it, as Yip and Dennis suggest, a question of retaining in the adult newt a faculty which is normally confined to development, or is it perhaps related to the way that foreign and original synapses are distributed along the length of the muscle fibres? It would be interesting to determine whether the foreign nerve in Yip and Dennis's study can become the correct one when the reciprocal experiment is performed in its own muscle. The most helpful insights, however, will probably come from an analysis of the mechanism. One would like to know if the interaction has to occur between synapses that are relatively close (of the order of microns) or whether it can occur over distances of the order of millimetres. If the latter is so, one might suspect that the muscle fibre plays a direct role in the process. An informative experiment could be to take a muscle that is only partially re-innervated by the original nerve, and determine if the suppressive influence can act on foreign synapses present on adjacent fibres that were not re-innervated. A second major question, which I have considered above, concerns the effect of the interaction on the anatomy of the suppressed synapses. If we knew the answers to these two questions, then the number of plausible mechanisms would be reduced.

I do not think that we can distinguish these possibilities with the available evidence, but some should be clearly excluded by an anatomical study. Other questions, such as the role of activity in the two nerves, and in the muscle fibre, also seem approachable. This whole problem illustrates the advantage of working on the neuromuscular junction—a preparation with which one can ask a basic question about synaptic function, and hope to answer it at several levels. Furthermore, there is every reason to believe that these answers will prove relevant to the experimentally less accessible situation of synapses in the central nervous system.

Helping hand to nuclear technology

from Andrew Holmes-Siedle

AS Townsend commented in a recent review (*Nature*, **258**, 293; 1975), in many universities, solid-state physics is taught in too ideal a fashion, as if real solids consisted of perfect arrays of atoms. While the existence of defects may be acknowledged, their importance or impact is not given its true weight. As a result, physicists often see the physics of defects as a fringe area of little importance. The commonly held belief is that colour centres and other invisible point defects are academic curiosities, confined to those near-useless materials, the alkali halides. So the report of a distinguished working party of the American Physical Society (Vook *et al.*, *Rev. mod. Phys.*, **47**, Suppl. 3; 1975) which states precisely the importance which small defects could have in nuclear technology and even makes a reasoned estimate of the possible cost of ignorance in this branch of physics is especially welcome.

The task at hand is to find out what will happen to the materials that constitute the structures and containment systems for the massive nuclear reactors (fission and fusion) which will supply most of our energy in two decades' time. Over the anticipated 20-year life of these reactors, some parts will receive fast neutron fluences in the range 10^{23} to 10^{22} fast neutrons cm^{-2} . The effect of this irradiation will be that every atom will have been moved from its lattice position about a hundred times and this constitutes a very serious case of radiation damage. The costs of ignorance in this field, it is claimed, could be as high as \$10,000 million over the coming decade.

One might ask whether all the basic radiation damage problems have not been worked out in the first development of power reactors. But the neutron flux levels envisaged in future fission reactors (fast breeder power reactors) are orders of magnitude higher than for present ones; the fluxes of particles expected to irradiate the walls of fusion reactors, moreover, are even higher and more varied in type. Also, past research in radiation damage, though intensive, has only scratched the surface of a field of great depth and subtlety. In fact, present basic knowledge of defects is too superficial even for the needs of today's reactor designers.

The working party's estimate of future costs due to ignorance stem from a vision both of the immense overdesign which often results when

knowledge is inadequate and of the inefficiency which results from "large-scale specific R and D testing imposed by limitations of basic knowledge". It sets out to delineate and dispel this limitation with detailed recommendations for research work which, although not costed in the report, could very probably be done for much less than \$10,000 million.

In the report, entitled *Radiation Effects on Materials* and one of a series on "Physics Problems relating to Energy Technology", the radiation effects field is split into ten main aspects. The ideas and numbers were hammered out during a ten-day seminar at Brookhaven. The problems of the different classes of defects (small, extended and so on) are first discussed; then the complexities of theory and simulation of damage by neutrons and some special effects (hydrogen, helium, surface effects) are described mainly in relation to metals. Finally, the three other solid state forms of greatest interest are discussed—semiconductors, superconductors and insulators. Reactor technology has its own section. In each case, a succinct description is followed by reasoned recommendations, while the main object—to point to specific areas in which the physicists can help the reactor designer—is kept in mind throughout.

The complexity of some of the physical problems can be illustrated by the void problem (see, for example, Bullough and Nelson, *Physics in technology*, **5**, 29; 1975). At temperatures in the 500–800 °C range, the conventional recombinations between atom vacancies and interstitial displaced atoms are disturbed, defects aggregate instead of recombining and the material swells. A volume increase of 15% is not unusual. The aggregated defects can be observed as interstitial loops, which are not new to us but the vacancies, at high damage levels, form a structure which is completely new to physics, namely an aggregate of vacancies large enough to form a visible void in the lattice. Such a structure introduces very great complications into the physical model of damage, since it represents an internal surface. Among other practical consequences of its existence, the new surface can act as a sink for impurities and for gaseous transmutation products trapped in the material. Their presence will also affect strength, but it is hard to predict exactly what effects will result from a

given change in the composition of a material. Without a predictive method, physicists cannot help the materials engineer to develop or select more radiation tolerant materials.

The economic usefulness of the physicist here is in his ability to change what might otherwise be a near-random search for better materials into one in which principles for the needed improvement of materials can be stated and tested in the least expensive way. Once the best possible material has been developed, the physicist's power to predict its properties after 20 years of bombardment may then tip the scales in the question of the reactor's economic viability. Without these data the designer might, using a necessary conservatism, design a cart-horse of a reactor, immensely strong mechanically but poor at making power. There will also be a need, not mentioned in the report, for a common scientific/engineering language which will facilitate communication between specialists.

Because a magnetically-confined fusion reactor, unlike a uranium reactor operates at high voltage, thermonuclear power reactors (if they are ever developed) will require a variety of strong, long-lived insulators. The report dwells on our knowledge of radiation damage in the alkali halides, and our lack of it in the oxides. But it missed an excellent argument for extra effort in these fields. Whereas the damage problem is greatest in the structural metals, the techniques by which damage can be understood in conductors are more limited than those which can be employed for dielectrics, mainly because optical and magnetic resonance spectroscopy cannot be used. There seems, however, to be a good chance that models for damage processes which are common to both types of material (for example the escape of defects from damage spikes, their diffusion in the lattice and their addition and loss from voids and loops) could be developed to a high level of sophistication using dielectric samples and the models could then be corrected as necessary for the case of metals and applied to the serious problem of degradation in strength.

Another little-known fact which is clearly documented in the report is the difficulty of simulating in a short time the 20-year torture which reactor structures will receive. Neutron sources of the required flux and energy (14 MeV for fusion, and about 1 MeV for

fission) do not exist for the damage range (up to say 500 displacements per atom) which we need to explore. For many purposes, beams of heavy ions can simulate neutron damage well enough. For example, about 10^{16} 7.5 MeV tantalum ions cm^{-2} in vanadium foils at 700 °C produce voids which are not dissimilar to those produced by 10^{22} fast neutrons cm^{-2} ($E > 1$ MeV). The latter irradiation would take months in the most powerful fission reactor while the former takes hours or minutes in an accelerator.

The working party makes two pleas

—that the solid-state physicist should be brought more intimately into the nuclear programme and that, to save money, money will have to be spent on simulation machines and basic research. Good arguments are offered as to how overdesign through ignorance could undermine economic viability. Now, it is to be hoped that the agencies planning our future in the energy field will consider this message and implement it. Perhaps a similar call by European scientists, especially confirming the financial theme, would drive the point home to their own governments. □

Filamentous phage assembly

from P. J. G. Butler

THE filamentous bacteriophages of *Escherichia coli* are a family of viruses consisting of a single-stranded circular DNA molecule, encapsidated into a very long, narrow and flexible particle (reviewed by Marvin and Hohn, *Bact. Rev.*, **33**, 172; 1969). Unlike other bacteriophages, they are neither lysogenic nor do they establish a lytic infection, but rather a productive infection resulting in the slow secretion of progeny virus during a comparatively long period. This poses particular problems during virus release, which occurs without either lysis of the cell membrane or the budding off of regions of this membrane as with lipoprotein coated virions. The solution lies in a series of specific interactions between the viral coat protein and the cytoplasmic membrane of the cell, which are now beginning to be described.

The virus particles contain predominantly a single type of coat protein of about 5,000 daltons. Marvin and Wachtel (*Nature*, **253**, 19; 1975) have analysed the X-ray scattering from oriented gels of one of these bacteriophages (Pf1) and were able to fit this with a model in which the protein molecules are fully α helical and are themselves arranged helically in the particle, with the axes of the α helices lying almost parallel to the particle axis. This results in a tight interlocking of the coat protein molecules, giving a high degree of protection to the DNA while allowing flexibility of the particle. The sequences of the coat proteins are compatible with such a structure and Nakashima *et al.* (*Nature*, **253**, 68; 1975) showed that the hydrophobic central region could readily form stable α helices which could interact in the appropriate way, while there is a slight excess of basic residues at the C terminus, which could be at the inside of the protein coat and help to neutralise the DNA phosphate residues.

Although the attachment of the phage to the host cell, on or near the few molecules of attachment protein per particle, appears to be relatively straightforward, the transport of the viral DNA both into and out of the cell through the cytoplasmic membrane involves more complex mechanisms. The DNA uptake during infection requires DNA synthesis in the cell, during which Marco, Jazwinski and Kornberg (*Virology*, **62**, 209; 1974) found the single-stranded viral DNA to be converted into the double-stranded replicative form (RF). As the DNA is drawn into the host cell, the viral coat protein becomes incorporated into the cytoplasmic membrane and Smilowitz (*J. Virol.*, **13**, 94; 1974) showed that it is reused during the subsequent maturation of progeny virions, being incorporated dispersively into their protein coats, and thus leading to an economy of protein synthesis.

After the establishment of the infection, with viral protein and DNA synthesis in the host cell, the assembly of progeny virus occurs. Initially the

new virion DNA strands are assembled into a nucleoprotein complex with the protein product from gene 5, which is not the virus coat protein. These nucleoprotein complexes are again long, flexible particles and the protein molecules are probably helically arranged. However, Pratt, Laws and Griffiths (*J. molec. Biol.*, **82**, 425; 1974), while demonstrating their formation *in vivo*, found that these particles are much less stable than the mature virions, in which the gene 5 protein has been replaced by the coat protein. They suggested that the intracellular coat serves to protect the DNA until it acquires its final coat while being extruded through the cell membrane, to which the coat protein has already bound, and then recycles onto fresh DNA.

The site of the interaction between the phage coat protein (of M13) and the cytoplasmic membrane has been examined by Wickner (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 4749; 1975). He found that ^{125}I -labelled antibody raised against detergent-solubilised coat protein would bind specifically to both the intact phage and the coat protein in the membrane. Using this antibody to assay the coat protein, initially of the infecting virus, he confirmed that RF formation was necessary for the transfer of the protein from the incoming virus to the cytoplasmic membrane of spheroplasts, this process taking about 10 min and leaving the protein available for reuse in progeny virion assembly. After about 40 min, further coat protein begins to accumulate in the membrane as it is synthesised. Both the parental and the newly synthesised protein are similarly located in the cytoplasmic membrane, with the antigenic sites only detectable on the outside of the membrane and being masked if the spheroplasts are inverted by sonication. This observation implies that the protein must occupy a position in the membrane irrespective of whether it is inserted from the outside, during initial infection, or from the inside by *de novo* synthesis. Such rapid transport across the membrane is in marked contrast to the slow rate of flipping of phosphatidyl choline, which Kornberg and McConnell (*Biochemistry*, **10**, 1111; 1971) showed to have a half-time for equilibration of about 6.5 h, suggesting that some specific mechanism may be involved for its insertion through the membrane.

Further evidence for specificity in the insertion of M13 coat protein into the membrane, whether from the inside or the outside, comes from Wickner's observation that the antibody reaction is similar with either spheroplasts or intact cells. Since these cells do not release their periplasmic enzymes, the coat protein must have some special



A hundred years ago

SEVERAL letters have appeared in the *Daily News* calling attention to the fact that on Sunday week red snow was observed to have fallen in several parts of the country—at Forest Hill and Streatham in the south of London, at Reading and at Thurston in Norfolk. This phenomenon was observed in ancient times, and is referred to by Pliny; in modern times it has been frequently observed in all parts of the world, and is familiar to Arctic explorers. The phenomenon is generally ascribed to the presence of an algae, *Protococcus nivalis*.

from *Nature*, **13**, March 23, 415; 1876

localisation which allows access of the antibody.

In spite of the similar antigenic reaction of the coat protein, whether in the intact phage or incorporated into the cell membrane, Nozaki *et al.* (*Nature*, **259**, 335; 1976) suggest that the protein conformations show substantial differences. They examined the circular dichroism spectra of intact phage (f1) and of its coat protein in aqueous solution with various detergents or dissolved in micelle-forming lysolipids. The spectrum of the protein in the intact virus is quite unlike that of other proteins in simple aqueous solution, for while it shows the peak wavelengths characteristic of an α -helical chain, the intensities of these peaks are surprisingly different. This unusual intensity distribution can be explained by the bunching of the chromophores which will occur in the packing of more than 2,000 protein molecules into a single virus particle. With this correction, the spectrum is consistent with the entirely α -helical conformation for the protein previously suggested by Marvin and his colleagues. The spectra of the amphiphile-solubilised protein are all similar to each other and different from that of the protein in the virus in a way which suggests less than 50% α -helix content. This change is not, however, consistent with a significant part of the polypeptide having a random coil conformation and Nozaki *et al.* suggest that the transition may be from α helix to β conformation. Such a conformation would be compatible with the observed dimeric state of the protein in detergents and with the amino acid sequence around residues 26 to 36.

This structural change does not essentially contradict the similar antigenicity, since Wickner cites preliminary evidence that the antigenic site is in the first eight amino acid residues from the N terminus. In the model proposed by Marvin and Wachtel, this region of the polypeptide chain is on the surface of the virus particle and it is not the region suggested to undergo the structural transition. But this gross rearrangement of secondary structure is unique among the mechanisms so far proposed for virus assembly and the interest of this phage protein-cell membrane system should encourage further study. □

Kelp, abalone and sea otters

Robert M. May

BEFORE being all but exterminated by fur traders in the 18th and 19th centuries, the sea otter (*Enhydra lutris*)

occupied a range around the north-east rim of the Pacific Ocean from the northern Japanese archipelago, through the Aleutian Islands, and along the coast of North America as far south as Baja California. Natural populations have persisted mainly in remote parts of the original range, particularly in the Aleutians and parts of southeastern Alaska. Recently, however, with the enactment of protective legislation and the reintroduction of sea otter populations off the coasts of Oregon, Washington, British Columbia and parts of central California, the species is making a comeback along the Pacific coast of North America.

Although this fact is gratifying to conservationists, it is less pleasing to those involved with abalone fisheries. Adult sea otters in captivity have been found to eat 20–25% of their body weight daily, and benthic invertebrates (such as sea urchins or abalone) are the main component of this diet. Several studies have shown a decrease in sport and commercial abalone fisheries following the arrival of sea otters into previously unoccupied areas in California.

The dominant role played by sea otters in determining the structure and dynamics of nearshore communities is demonstrated by several recent ecological studies. Estes and Palmisano (*Science*, **185**, 1058–1060; 1974) compared islands in the western Aleutians where sea otters have occurred naturally for at least 20–40 years (the Rat Island group) with islands lacking otters (the Near Islands). They found many floral and faunal differences between the lower intertidal marine communities on these islands: the Rat Islands have an almost complete mat of benthic brown algae (kelp), with both sessile filter-feeding invertebrates (barnacles and mussels) and motile herbivorous invertebrates (sea urchins and chitons) being inconspicuous, small and scarce; the Near Islands are heavily grazed by dense populations of sea urchins and chitons. The density of barnacles, mussels, sea urchins and chitons, respectively, averaged around 4.9, 3.8, 8, and 1 m⁻² at the Rat Islands, and 1,200, 720, 78, and 38 m⁻² at the Near Islands.

Dayton (*Fish. Bull.*, **73**, 230–237; 1975) has followed this with a very detailed study of the competitive interactions between three kelp canopy "guilds" in a community at Amchitka Island, Alaska, in which herbivorous invertebrates have been largely removed from shallow waters by sea otters. This work confirms that sea otters are extremely important in maintaining the structure of shallow water algal communities, and tempts Dayton to the evolutionary speculation that, by reducing herbivorous preda-

tion, the sea otters permit a competitive differentiation of niches whereby the several kelp species coexist.

All these people are in agreement that, as sea otters become more established along the California coast, there should be a pronounced increase in the abundance of nearshore kelp. Dayton cautions against accepting this as evidence of the "natural" order of things, and he emphasises that the once-abundant giant sea cow (*Hydrodamalis gigas*) must have had important consequences to the kelp populations, that undercut any present day speculation as to the evolutionary consequences of kelp competition. Indeed, Dayton conjectures, it is likely that by consuming invertebrate herbivores (particularly sea urchins) "the sea otter was indirectly responsible for the high productivity of large algae necessary to maintain the sea cow populations." This conjecture is supported by the overlap of otter and sea cow populations in the Pleistocene.

This story is typical of many, where layer after layer of complication unfolds as one seeks to elucidate the structure of the pristine ecosystem, as it was before first sea cows and then sea otters were removed. Dayton gives a general warning against drawing evolutionary morals from partially reconstructed systems, because one or more crucial species may have become extinct at a time which seems distant in human memories, but is recent on an evolutionary time scale. □

Glaciology's grand unsolved problem

from J. Weertman

OVER the past two decades our understanding of the behaviour and the motion of glaciers, ice shelves, and ice sheets has progressed and increased very nicely. We know enough now to recognise a grand glaciological problem that remains to be solved. The West Antarctic Ice Sheet is this problem. Actually it is a set of inter-related problems. The base of this ice sheet over a major part of its area is 0.5 to 1 km below sea level. A major fraction of its bed would remain below sea level if the ice sheet were removed and isostatic rebound took place. How then did this ice sheet form? Why does it remain in existence? Is it growing or disintegrating at the present time? The ice in one half of the West Antarctic Ice Sheet is draining primarily through very fast moving ice streams into the Ross Ice Shelf. These ice streams apparently are not centred over any deep, fiord-like channels cut

into the bedrock, and therefore could not have existed in their present position for any great length of time. The velocity of ice in the ice streams, about 1 km yr^{-1} , is comparable to that of the ill-understood surging glaciers. (Surging glaciers temporarily move, over a 1 to 2-yr period, at velocities that are one to three orders of magnitude larger than a normal glacier velocity.) Why do very fast moving ice streams form at the periphery of the West Antarctic Ice Sheet? Why do they move so swiftly? Is their existence a premonitory phenomenon to a large scale surge of the whole ice sheet? Are the ice streams the start of a process that may cause the West Antarctic Ice Sheet in the near future to flow very rapidly and to discharge about one-third to one-half of its total volume of ice into the oceans over a short time period of the order, say, of 100 years?

Geological evidence that was studied by Denton, Armstrong, and Stuiver (in *Late Cenozoic Glacial Ages* (edit. by Turekian) 267, Yale University Press, 1971) and by Denton and Borns (*Antarctic J.*, 9, 167; 1974) indicates that before 10,000 years ago the West Antarctic Ice Sheet was much larger and covered the area, now below sea level, presently occupied by the Ross Ice Shelf. The Ross Ice Shelf, although about 300 to 500 m thick, is afloat. Thus, the edge of the ice sheet has retreated about 1,000 km with an average rate of retreat of the order of 100 m yr^{-1} .

It is not likely that this large scale retreat was produced by any change in the climate. There is at present a net accumulation of snow on top of both the West Antarctic Ice Sheet and the Ross Ice Shelf; in fact, the accumulation rate is larger on the ice shelf than on the ice sheet. Studies of the ice cores from drill holes in both the ice sheet and the ice shelf have produced no evidence at all of any long lasting period of widespread ablation that would have caused the shrinkage of the West Antarctic Ice Sheet. A more likely explanation of the retreat is that it was caused by the large rise in sea level, 100 to 130 m, that occurred when the ice sheets in the northern hemisphere melted at the end of the last ice age. According to theory (Weertman, *J. Glaciol.*, 13, 3; 1974) the size of an ice sheet whose base is below sea level is a very sensitive function of changes in sea level. Whether the ice sheet can even exist over a long period of time is dependent upon the sea level.

The geological evidence of fossil coral-reef terraces in the Barbados (Broecker *et al.*, *Science*, 159, 297; 1968) indicates that 120,000 years ago the sea level was 6 m higher than it is today. Old coral-reef terraces in New Guinea (Chappell, *Geol. Soc. Amer.*

Metallic glazes

from Robert W. Cahn

A NUMBER of alloys based on transition metals—notably nickel and iron—can be made in the form of glassy ribbons by ultra-rapid quenching of a molten jet on a rapidly spinning roller (see *Nature*, 252, 100, 1974; 259, 271; 1976). Iron-based glassy ribbons containing chromium have the further distinguishing feature of being extremely corrosion resistant. These materials, however, have to be in the form of thin (0.05 mm), narrow (1–2 mm) ribbons, and there is not a great deal that the most ingenious designer can do with them. Much interest therefore attaches to a report of a novel method that allows the surface of bulk material to be converted into glass without affecting the interior.

A group of metallurgists at the United Technologies Research Center in Connecticut, led by Bernard H. Kear and Edward M. Breinan have described to the American Society for Metals how a continuous carbon dioxide laser can be used to “glaze” the surface of nickel or cobalt-based alloys. The Center is linked with a factory that makes aircraft engines, and the implications of the choice of alloys are clear enough.

A finely focused beam from the CO_2 laser is passed over the surface of the bulk material; the surface is rapidly melted and as rapidly refrozen as the hot spot moves on. A cooling rate exceeding a million degrees per second was claimed, during resolidification, and this suffices to generate a glassy (non-crystalline) phase at the surface. The alloy compositions used are not specified: it would be interesting to know whether they contain metalloid solutes such as P, C, B, Si, as do the alloys used to make glassy ribbons.

Bull., 85, 553; 1974) also support this conclusion. A 6 m rise in sea level requires the total removal (and melting) of ice from an ice mass of about the size of the West Antarctic Ice Sheet or the Greenland Ice Sheet and its emplacement into the world's oceans. This observation of a 6 m higher sea level 120,000 years ago suggests that the West Antarctic Ice Sheet can largely disappear under conditions that do not lead to the destruction of the East Antarctic Ice Sheet. The destruction of the latter ice mass would have raised the sea level by another 40 to 50 m.

Terence Hughes of the University of Maine has strongly advocated the

It is particularly worth noting that the underlying crystalline grains do not act as nuclei for the resolidifying surface film.

The investigating team reports that the surface glaze is harder than the base alloy, and adds that it is “potentially more capable of resisting corrosion than the original alloys”: the cautious choice of words leaves one uncertain whether the potential had been realised.

This is not the first time that a laser has been used to effect rapid quenching from the melt. Laridjani, Ramachandrarao and Cahn (*J. Mater. Sci.*, 7, 627; 1972) used a stationary beam from a pulsed ruby-laser to produce metastable crystalline phases on the surface of Ag-Ge alloys, and Elliott, Gagliano and Krauss (*Metallurgical Trans.*, 4, 2031; 1973) used a pulsed neodymium laser to create a continuous series of solid solutions in a range of Cu-Ag alloys which are only partially miscible in equilibrium. The United Technologies metallurgists have improved on this earlier work in two ways: they examined alloys based on transition metals which, unlike copper and silver-based alloys, are apt to form glasses on melt quenching, and they used a moving beam from a continuously acting laser to scan the surface. What is not clear from the report is whether it was feasible to make a series of overlapping passes in such a way to cover a surface entirely with a glazed layer. If this can be done, the industrial potential is very considerable; it should in principle be possible to “coat” even the inside of a vessel with the aid of a guided moving mirror. There is also potential for the design of wholly or partially glazed bearing surfaces.

view (and put forward supporting evidence) that the West Antarctic Ice Sheet is still disintegrating (*J. geophys. Res.*, 78, 7884; 1973). He thinks that the rate of retreat of the edge of the ice sheet where it joins the Ross Ice Shelf is of the order of 70 m yr^{-1} . This rate of destruction of the ice sheet would cause the sea level to rise with a velocity of the order of 0.5 mm yr^{-1} . The long term increase in mean sea level occurring now has been estimated from various measurements to be 1 mm yr^{-1} (Lisitzin, *Sea Level Changes*, 183, Elsevier, Amsterdam, 1974). Thus, a slowly occurring destruction of the West Antarctic Ice Sheet could account

for the present rate of rise of the mean sea level.

It would be difficult to measure a yearly shift of the position of the edge of the West Antarctic Ice Sheet of the order of only 100 m. The thickness of ice at the junction of this ice sheet with the Ross Ice Shelf is at least 500 m. The position of the grounding line certainly cannot be found with an error limit smaller than the ice thickness. It seems clear that a great deal of field data must be collected on such things as ice thickness on both the Ross Ice Shelf and the West Antarctic Ice Sheet before the question can be answered whether this ice sheet is disintegrating or growing and at what rate.

Many data have been collected now, at least for the Ross Ice Shelf (see Robin, *Nature*, **253**, 168; 1975). These and new data have been analysed by Thomas (*Nature*, **259**, 180; 1976). He thinks that at the grounding line the Ross Ice Shelf is thickening at the high rate of 1 m yr^{-1} . This rate of thickening then leads to his startling conclusion that the position of the edge of the ice sheet at least at one location is advancing at the very fast rate of 1 km yr^{-1} . Thomas cautions that this result is not expected to be typical. He believes that the apparent current thinning of the ice sheet nearer its centre is a delayed response to an earlier retreat of the grounding line.

So now a controversy exists about whether the West Antarctic Ice Sheet is growing or retreating and whether perhaps it is starting to surge. It will take a lot of new field data from the West Antarctic Ice Sheet as well as theoretical analyses to settle the question. □

Errors in lipoprotein metabolism

from a Correspondent

FAMILIAL hypercholesterolemia is an inborn error of metabolism in which affected individuals show a marked elevation in plasma cholesterol, progressive coronary, cerebral and peripheral vascular occlusive disease associated with accumulation of cholesterol in atheromatous plaques. Death from myocardial infarction often occurs before the age of 30. Cholesterol accumulation, which is the most striking biochemical change seen in these patients could arise because of increased synthesis or because of a failure of response by the liver to feedback controls on that synthesis by plasma levels of cholesterol. Some recent

findings have gone a considerable way to defining at least one cellular abnormality carried by some patients with this disease. It does seem, however, that the clearly identified clinical phenotype may hide several different abnormalities at the cellular level and therefore what I report here refers only to a proportion of affected individuals. Nevertheless, the recent results of Brown, Goldstein, Steinberg and their colleagues provide exciting pointers to one probable cause of this disease.

A genetic abnormality characteristic of the disease trait in some instances is carried by skin fibroblasts established from biopsy material. It was shown by Brown and Goldstein (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2162–2166; 1973), that normal skin fibroblasts exposed for about a day to foetal calf serum made deficient in lipoproteins accelerate cholesterol biosynthesis by activation of a key enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase. The normal fibroblasts respond, through regulation of the level of activity of the enzyme, to inhibition of cholesterol biosynthesis by addition of lipoprotein. The response is rapid, occurring over a few hours. In contrast to the normal behaviour, skin fibroblasts from homozygous patients with hypercholesterolemia do not respond to lipoprotein and cholesterol synthesis continues unabated in lipoprotein-supplemented serum (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2804–2808; 1973). Brown and Goldstein reasoned that the effect of lipoprotein on the fibroblasts must be mediated firstly by attachment of lipoprotein to surface receptors followed by internalisation of lipoprotein and a terminal effect on cytoplasmic events involved in cholesterol biosynthesis. On the basis of binding data of lipoprotein to normal or hypercholesterolemic fibroblasts, these workers proposed (*J. cell. Physiol.*, **85**, 425–436; 1975), that the specific block was in the lack of high affinity surface receptors in the latter cells. The binding data were, however, collected by treating cells with labelled lipoprotein at 37°C , that is, under conditions in which other events, quite apart from the initial binding of lipoprotein molecules to their specific cell receptors, would be expected to take place. It seems probable that both binding and internalisation of lipoprotein molecules would occur, by analogy with the well studied capping and envelopment of lymphocyte surface immunoglobulin after combination with bivalent antibodies at elevated temperatures but not in the cold (see Raff, *News and Views*, **259**, 265–266; 1976). The binding data of Goldstein and Brown probably sums the levels of lipoprotein bound at the cell surface and intracellular lipoprotein, and it becomes difficult to distinguish a block in in-

ternalisation of bound lipoprotein molecules rather than in the binding process itself.

Stein *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **73**, 14–18; 1976) have reassessed this problem, and propose that it is indeed the internalisation step that is grossly defective, at least in the cells studied by them. They carried out experiments in which the binding of radioiodinated lipoprotein to fibroblasts was measured at 0°C . The differences compared with normal fibroblasts were small at best, not greater than about two-fold. Essentially all the bound radioiodinated lipoprotein was released from both normal and hypercholesterolemic cells by mild trypsin treatment without disruption of the cells. Therefore, it was concluded that these measurements refer accurately to cell surface binding and the latter cells seem to be almost normal in this respect. Of course, it is certainly possible that this relatively small decrease in lipoprotein binding may be important in the physiological response of the cells to lipoprotein, for example by the specific loss of a class of cell surface receptors important for the subsequent events of lipoprotein action on cholesterol synthesis. But by far the most striking effect was seen in the amounts of labelled lipoprotein present inside the cells after incubation at 37°C . This was measured after treatment with trypsin to remove surface located molecules. The normal cells contained from 14–115 times as much labelled material as the hypercholesterolemic cells, measured under the same conditions.

What is the nature of the “receptors” for lipoprotein in the fibroblast surface membrane? This is not known with any certainty, nor indeed whether there are specific “receptors”. The lipid moiety of the lipoprotein could react in some way directly with other lipids of the cell membrane. It seems, however, that the sites at which lipoprotein molecules become associated with the membrane may be critical for exertion of the biological effect on cholesterol biosynthesis, implying recognition of these sites by lipoprotein. At high concentrations of endogenously added lipoprotein, the level of internalised material in hypercholesterolemic fibroblasts may rise to comparable levels to those found in normal fibroblasts exposed to more moderate concentrations. Yet there is no accompanying inhibition of sterol synthesis. Goldstein *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1092–1096; 1975) therefore suggest that the interaction with specific receptors on the membrane is a necessary step in the overall control mechanism, and presumably this interaction is also critical in internalisation. If this turns out to be the case, the identification of the specific membrane components responsible is

clearly of great importance in defining the ultimate defect in hypercholesterolemia. □

Who killed T7?

from Rich Condit

FOR people interested in control of translation, for those interested in phage protein-host membrane interactions, or for people who simply enjoy molecular whodunnits, the history of research into the abortive infection of male *E. coli* by bacteriophage T7 makes a rather good story.

T7 is a virulent phage which grows only on female *E. coli* (Makela *et al.*, *Ann. Med. exp. fenn.*, **42**, 188; 1964). When male *E. coli* are infected with T7, the cells are killed as is normal in a T7 infection, but no cell lysis occurs and no phage are produced. The initial probe into the molecular biology of the abortive infection indicated that the phage DNA entered the male cell and was completely transcribed, but that only early T7 proteins were produced. In addition, the wild type F factor (PifA⁺B⁺) could be mutated so that the block in translation was either partially (PifA⁻B⁺) or completely (PifA⁻B⁻) relieved (Morrison and Malamy, *Nature new Biol.*, **231**, 37; 1971). The hypothesis which emerged to explain these phenomena maintained that during a normal infection, T7 makes two translational control factors, each required for translation of a discrete class of late T7 mRNAs, and that the F factor encodes two proteins, each of which specifically sabotages one of the T7 coded factors. Because of this hypothesis, T7 has been regarded as a model system for studying translational control of protein synthesis.

In the past few months, several reports have appeared which render most of the translational control hypothesis untenable, and suggest alternative explanations for the abortive infection. One of the most important facts emerging from these studies is that, in contrast to initial findings, it now seems that T7 mRNA synthesis is abnormal in infected male cells. Analysis of phage RNA synthesis in male cells by pulse labelling, by hybridisation, and by translation reveals that although synthesis of early T7 mRNA is normal and synthesis of some late mRNA is close to normal, many late phage RNAs are synthesised in substantially reduced amounts, or not at all (see for example Britton and Haselkorn, *Virology*, **67**, 264; 1975; Condit and Steitz, *J. molec. Biol.*, **98**, 31; 1975; Young and Menard, *J. molec. Biol.*, **99**, 167; 1975). In fact, it seems

that T7 infection of male cells results in a generalised paralysis in synthesis of macromolecules (RNA, protein, and DNA) rather than simply a block in protein synthesis. In short, the infection appears normal until late phage protein synthesis just begins (by which time some late RNAs have been made in substantial amounts) and then the infection simply dies.

Attempts to reconstruct the translational control hypothesis *in vitro* have met with only partial success. First, ribosomes from uninfected male and female cells are identical with respect to their ability to synthesise T7 proteins. Second, ribosomes from uninfected cells are fully capable of translating late T7 mRNAs, demonstrating that no phage-induced alteration of host ribosomes is necessary for translation of late T7 mRNA. Finally, though some groups have been unable to detect any significant defect in ribosomes extracted from T7 infected male cells (Condit and Steitz, *op. cit.*; Ponta *et al.*, *Eur. J. Biochem.*, **59**, 261; 1975; Young and Menard, *op. cit.*), others have observed that these ribosomes are reduced in activity in an *in vitro* system by a factor of 3–5, relative to ribosomes from uninfected cells or cells undergoing a productive T7 infection (Yamada and Nakada, *J. Virol.*, **16**, 1483; 1975; Blumberg *et al.*, *J. Virol.*, **17**, 94; 1976). The defect is generalised, that is, the ribosomes from infected male cells are defective regardless of the mRNA used in the test, the defect seems to be reversible by addition of a trypsin-sensitive, non-dialysable fraction from cells containing healthy ribosomes (Blumberg *et al.*, *J. Virol.*, **17**, 94; 1976), and the defect becomes apparent during infection of wild-type male cells at approximately the same time that everything else goes wrong (Yamada and Nakada, *J. Virol.*, *op. cit.*).

In summary, what remains of the translational control hypothesis is that the ribosomes in male cells seem to sustain a certain amount of damage as the T7 infection aborts.

In the search for an explanation for the cataclysmic death of the infected cell, it has been discovered (Britton and Haselkorn, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 2222; 1975; Condit, *J.*

molec. Biol., **98**, 45; 1975) that at precisely the same time that macromolecular synthesis comes to a halt the T7 infected male cell becomes "leaky". There is a rapid loss of intracellular nucleoside triphosphates into the surrounding medium, the cells lose the ability to accumulate glutamine, and they become abnormally permeable to orthonitrophenylgalactoside. Furthermore, the appearance of these permeability defects depends upon both the presence of the F-factor gene(s) controlling the abortive infection, and on phage protein synthesis.

It is tempting to tack ribosome damage onto the list of things that might go wrong if membrane function is tampered with, and to formulate a "permeability hypothesis" to explain the abortive infection. Perhaps, because of the action of the F genes, a T7 protein misbehaves in the male cell and membrane function is rendered aberrant as a result. Macromolecular synthesis might be expected to cease because of a change in the intracellular ionic conditions and a decrease in the availability of small molecules. A recent study by Blumberg, Mabie and Malamy (*J. Virol.*, **17**, 94; 1976) however, questions the notion that membrane damage, ribosome damage, and the overall paralysis of macromolecular synthesis are causally related.

Blumberg *et al.* have compared leakage of ATP with activity of ribosomes using four types of T7-infected cells: F⁻ (productive infection, lysis at 30 min), PifA⁺B⁺ (wild-type male, infection aborts at approximately 8 min), PifA⁻B⁺ (mutant male, infection aborts at approximately 13 min) and PifA⁻B⁻ (mutant male, productive infection). They find that PifA⁻B⁻ cells behave exactly like F⁻ cells, that is they contain healthy ribosomes wrapped in healthy membranes. Whereas PifA⁺B⁺ and PifA⁻B⁺ cells show virtually identical leakage of ATP, however, ribosomes extracted at 8 min after infection are defective in PifA⁺B⁺ cells and normal in PifA⁻B⁺ cells. These observations indicate that the sudden paralysis of protein synthesis and the damage to ribosomes observed during infection of a wild-type male cell cannot be accounted for solely in terms of leakage of ATP. The PifA⁻B⁺ cell begins

Table 1

	F ⁻ (female)	Infected cell type PifA ⁺ B ⁺ (male)	PifA ⁻ B ⁺	PifA ⁻ B ⁻
Infection phenotype		Abortive	Abortive	Permissive
Phage protein synthesis	Permissive	Ceases ~	Ceases ~	Ceases with
<i>in vivo</i>	cell lysis (30 min)	8 min	13 min	cell lysis (30 min)
Ribosomes extracted at 8 min damaged?	No	Yes	No	No
Leakage of ATP at 8 min?	No	Yes	Yes	No

to leak ATP at 8 min after infection at the same rate as the infected PifA⁺B⁺ cell, and yet ribosomes are not immediately damaged, and protein synthesis continues for an additional 5 min.

Several matters need to be cleared up, therefore, before we will know whether or not the permeability hypothesis holds water. The results of Blumberg *et al.* may indicate that membrane damage is only a partial explanation for the abortive infection, or they may mean that leakage of ATP is an inadequate assay for the total amount of membrane damage sustained during infection of a wild-type male. Part of the difficulty in distinguishing between these two alternatives is that we do not know whether the Pif mutants really define two genes, or whether the PifA⁻B⁺ mutant simply contains a crippled Pif gene. In addition, it will help in evaluating the relative importance of ribosome damage and membrane damage when we know whether RNA synthesis is affected in the PifA⁻B⁺ male relative to the wild-type male, and whether or not ribosomes in the PifA⁻B⁺ male remain healthy even after the infection aborts.

A successful virus infection represents a very carefully balanced relationship between the virus and its host, which, in this case, has run amuck. There seems to be a T7 gene(s) which when expressed in male *E. coli* leads to permeability changes, and may ultimately be involved directly or indirectly in the observed paralysis of macromolecular synthesis and damage to ribosomes. Especially because of the implied interaction with the cell envelope, it will be interesting to discover the normal role of this phage gene during T7 infection of female cells.

Hormone and anti-hormone action at the target cell

from Tom Blundell

A Dahlem Workshop on Anti-hormones and the Target Cell was held in Berlin on February 16-20, 1976. The proceedings will be published by Dahlem Konferenzen.

SPECTACULAR advances in our knowledge have recently resulted from studies of hormone receptors. This is not only true for the membrane receptors of polypeptide hormones and catecholamines, but also for the intra-

cellular receptors of steroid hormones, vitamin D metabolites and thyroid hormone. Can these new insights into the field of receptors now be useful in the design of biological tools and therapeutic agents?

This question was central to the Fourth Dahlem Workshop on "Hormone and Antihormone Action at the Target Cell" held in Berlin recently at which endocrinologists with a wide range of interests in hormone receptor interactions gathered together with biophysicists, molecular biologists and protein biochemists.

Unfamiliarity with the new no-lecture format of the Dahlem meetings resulted in a hesitant beginning but the discussions soon warmed up with the help of the generous hospitality and efficient organisation of Silke Bernhard and her colleagues. In the following days the debate ranged over a number of difficult and neglected areas in the study of hormone receptor interactions, and demonstrated the need for discussions between the various scientific disciplines represented.

The term "antihormone" refers to all those substances that act to attenuate hormone-induced responses regardless of their mechanism. Thus mechanisms changing the affinity of the receptor for the hormone by direct competitive binding of antagonists or indeed by allosteric effectors are included, as well as factors which affect hormone availability at the target cell, loss of receptors, and regulation of receptor synthesis.

For steroid receptors a combined steric and allosteric model presented by M. Sherman (University of California Medical Center) seemed to be very useful. In this model, a dimeric receptor can exist in two conformational states, one of which is inactive because the receptor molecule cannot enter the nuclei or cannot bind to the proper genomic sites or is not released normally from the nuclei. A steroid that binds the inactive state is an antagonist, and one that binds the active state is an agonist. Any steroid that exhibits some affinity for both states is a partial agonist because under some conditions it will be a suboptimal inducer of the function studied, but in the presence of a pure agonist it may act as a partial antagonist. Thus the antagonism by progesterone of glucocorticoid-induced tyrosine aminotransferase in hepatoma cells results from the formation of a glucocorticoid receptor-progesterone complex which does not undergo translocation to the nucleus. On the other hand the ability of steroids to act as partial agonists is exemplified by the partial action of 11-deoxycortisol on sodium transport. In this system aldosterone binds exclusively to the active state while the 11-deoxycortisol binds

about half as well to the active and inactive states.

In the field of membrane receptors models seemed to be less in fashion and there was general emphasis on the need to define the macromolecular components more precisely. No hormone receptor or eukaryotic adenylate cyclase has as yet been purified although a characterisation using appropriate detergents as solubilising agents has been achieved for a number of membrane bound proteins such as Ca²⁺-ATPase, glycophorin and the acetylcholine receptor. C. Tanford (Duke University Medical Center) emphasised the need for using detergents such as Tween 80 with long hydrophobic tails which simulate the membrane bilayer but pointed out that a rather different detergent, for example deoxycholate, may be preferable for initial disruption and fragmentation of the membrane. The presence of detergent bound to a protein receptor does not interfere with the determination of molecular weight using sedimentation equilibrium. With pure preparations, the conformational state of occupied and unoccupied receptors can be defined and the design of antihormones carried out on a more rational basis.

Many hormones have not evolved to fit best the receptor of the same species. Although there is good species specificity with vasopressin analogues, salmon calcitonin has high activity in man when compared with human calcitonin. In a similar way insulin receptors from different species bind insulins from various animals with similar relative affinities. J. Roth (National Institutes of Health, Bethesda) suggested that the receptor proteins may be under strong evolutionary constraints because they have other functions essential to the membrane. Thus similar molecules may exist in the membranes of cells to which the hormone has no biological response and even in prokaryotic cells. This would provide an explanation for the reported activity of insulin and glucagon on bacteria and algae. It also suggests that it may be worthwhile to explore the possibility of synthesising molecules which are better agonists than the natural hormone.

Another observation that gave rise to much discussion was the recent report on the enkephalin peptides which appear to be the natural opiates. These will clearly stimulate the search for other natural peptides which bind drug receptors. Conversely it implies that a non-peptide molecule such as morphine can bind a peptide receptor, and that in the design of therapeutic agents which are peptide-hormone agonists or antagonists it should be possible to use analogues of a very different chemistry. □

articles

Geological and palaeontological background of Hadar hominid site, Afar, Ethiopia

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The hominid-bearing Plio-Pleistocene sequence of the west central Afar sedimentary basin in Hadar contains 120–140 m of fossiliferous lacustrine and lake margin sediments. The Hadar sequence is described and divided into four members. The mammalian fauna indicates an age of ~ 3.0 Myr which is confirmed under the Hadar formation by K–Ar determinations on a basalt.

THE Afar is a depression at the northern end of the East African rift at the triple intersection of the trends of the East African rift, the Red Sea and the Gulf of Aden (Fig. 1). After three field seasons by the International Afar Research Expedition (IARE), the site of Hadar in the Afar promises to become one of the richest fossiliferous Plio-Pleistocene localities associated with the East African rift system. Here we present the generalised geology and palaeontology of Hadar.

Earth Resources Technology Satellite (ERTS) photographs of the Afar reveal a region of predominantly dark tone, marked with lineaments and volcanoes. The rocks are mostly fissure erupted flood basalt, divided into a few long north trending grabens and horsts, with rhyolitic volcanic centres. Aside from the Tendaho graben containing Lake Abbe, the only sediment-filled area is the west central Afar sedimentary basin composed of Plio-Pleistocene deposits adjacent to the Western Ethiopian plateau¹ (Fig. 1). East of the sedimentary basin the flood basalt constitutes three fault blocks. Running from north to south these are: the Mille block, comprising the Guralle acidic centre overlain by the Amado sequence of the west central Afar sedimentary basin; the Magenta block; and the tilted Galalu block interbedded at its top with the sediments seen south of Hadar, and referred to as the Rougous sedimentary sequence. K–Ar ages have been determined regionally for these basalts^{2,3}, with most of them between 2–4 Myr.

The Plio-Pleistocene sedimentary basin is ~ 60 km wide and 150 km long. Much of this area is capped unconformably by an uppermost unit of Pleistocene gravels and some bedded sands containing Acheulean artefacts⁴. The underlying Plio-Pleistocene strata have already been referred to as the Central Afar Group⁵. The IARE has studied sequences of this group regionally at Amado, Gewane, Gueraru, Hadar, Haouana, Leadu, Ledi, and Meschelle (Fig. 1). Fauna from these sites were tentatively referred to a time range of 2–4 Myr in compari-

son with other eastern African sites⁵. Thus, the sedimentary basin was contemporaneous with the volcanism just to the east.

The Hadar Formation

WE refer to the area of Plio-Pleistocene sediments exposed in the drainage of the Awash basin in the tributaries of Gona, Sidi Hakoma, Kada Hadar, Ounda Hadar and south of the Awash River (Fig. 2) as the Hadar site of the west central Afar sedimentary basin. Operationally we refer to the exposed sequence in this area as the Hadar Formation, even though the upper and lower contacts of the formation have not yet been fully explored. The sediments represent lacustrine, lake margin and associated fluvial deposits, related to a Plio-Pleistocene lake which may have occupied nearly the entire sedimentary basin, at least periodically. The offshore deposits are predominately clay sediments with the remains of fish and ostracods. Nearer shore deposits are primarily silts or calcareous mudstones; shore deposits are sands with shallow cross-bedding. Fluvial deposits are represented by pebbly gravels and sands with steep cross-bedded channel fills cut in underlying strata. For the Hadar site generally, the sequence consists of a higher percentage of fluvial facies to the west near the Gona tributary, and offshore facies to the east near the Ounda Hadar, compared with Kada Hadar where the largest percentage of nearshore facies occurs. From Sidi Hakoma to Kada Hadar (Fig. 2), where the members of the formation have been defined, the sequence has ~ 45% clays, 25% silts, 25% sands and sandstone and 5% volcanic, carbonate and biogenic deposits. Most of the vertebrate fauna is found in the sands and sandstones. The frequent occurrence of complete fossil specimens as well as partially associated skeletons, and the general lack of taphic abrasion indicates a low energy depositional environment and a possible high rate of sedimentation.

The formation has an exposed thickness of 120–140 m in the Kada Hadar tributary. The sequence dips northwards by 2–4° with just a few steep normal faults trending 5°N–20°E with displacements of 5–40 m (Fig. 2). Several minor erosional unconformities can be seen in the Hadar Formation, but no major time gaps are suggested. The Hadar Formation has been conveniently subdivided into four members, separated by major tuff horizons as shown in the geological column (Fig. 3). A geological map (Fig. 2) and a generalised cross section (Fig. 4) are presented here. Each member in this tributary has three or

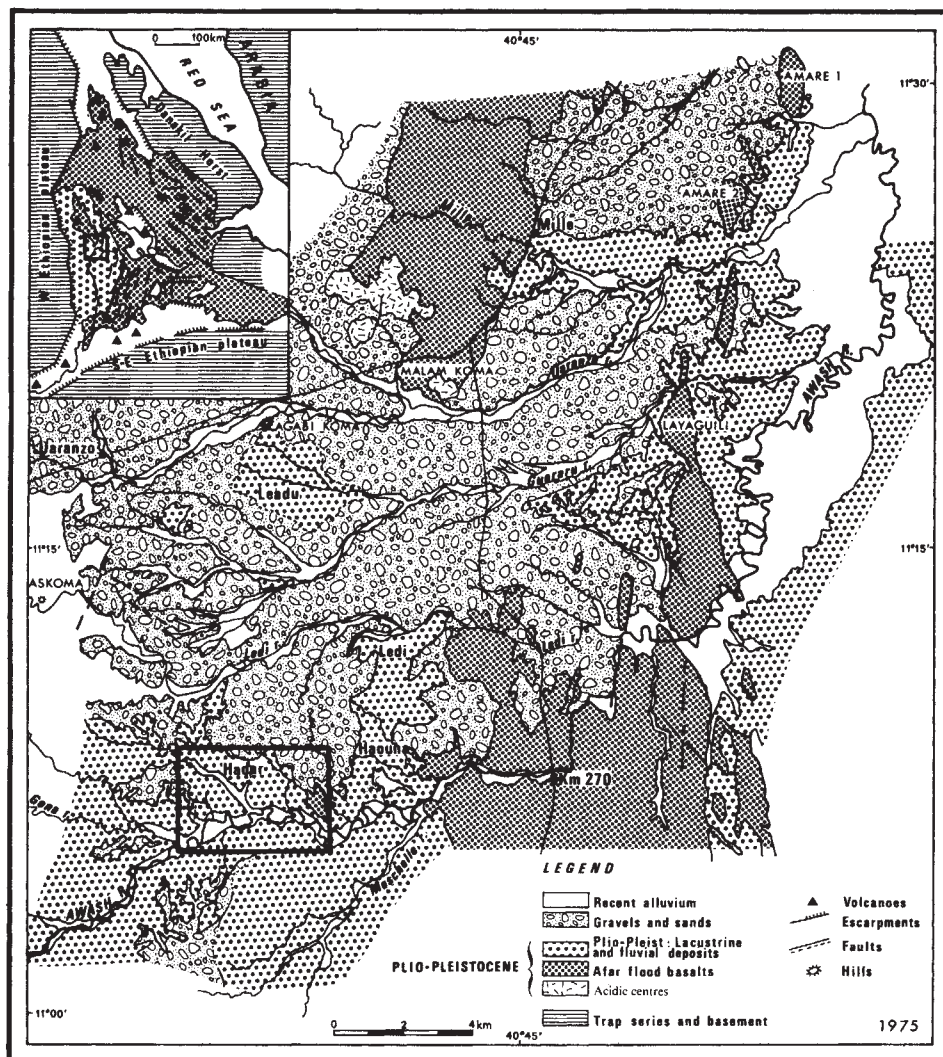


Fig. 1 General geological map of the north-western part of the west central Afar sedimentary basin (mapping with the collaboration of N. Page).

more submembers of alternating sands and clay-silt sediments. From the base to the top the members are: (1) the basal member (BM), 12 m exposed; (2) the Sidi Hakoma member (SH), with a series of white tuffs (SHT) at its base, 55 m thick; (3) the Denen Dora member (DD) with a series of thin tuffs (TT) in an extensive clayey marker horizon at its base, 25 m thick; and (4) the Kada Hadar member (KH), with a single tuff (KHT) at its base, 48 m thick.

Volcanics

In general the tuff horizons are laterally extensive and are nearly everywhere altered to a chalky white fine material. SHT and KHT have, however, been traced to palaeochannel fills in the underlying strata, where they are grey with a somewhat vitreous lustre, and consist of 95–98% glass shards derived from explosive pumice eruptions. Only the SHT channel fill shows closely spaced, steeply dipping cross bed sets in cut-and-fill troughs indicating a fast flowing current in the channel. The occurrence of fresh unaltered glass in the palaeochannels may arise from sorting of the coarsest glass, which resisted alteration.

At the eastern end of the Hadar site the Kadada Moumou subplateau is upheld by a single 1–4 m thick basalt flow, 60 m above the lowest exposures. The flow thins to the north-west where it ends. It lies over a section of largely clayey and silty sediments with two gastropod-rich layers (Fig. 4). Unconformably above the basalt is the post-Hadar Pleistocene gravel unit. In one locality, sediments of the Hadar Formation have been observed overlying the basalt. The Kadada Moumou subplateau geomorphic feature originated before the deposition of the overlying gravels when erosion was greatly slowed by

exposure of the very resistant basalt. Extending from the north-western edge of the basalt into the dipping Hadar sedimentary sequence is a local unconformity with a relief of 4–6 m. Above the unconformity are local steep bedded sands with numerous boulders of the basalt. These sands contain abundant Hadar Formation fauna and are overlain by a distinctive sand unit which is laterally extensive within the Sidi Hakoma member. There is no fault between the Kadada Moumou flow and the sedimentary sequence. Twenty metres stratigraphically above the basalt level in the sediments is the persistent horizon (TT) which marks the base of the DD member. The basalt is thus in the upper portion of the Sidi Hakoma member.

The basalt is vesicular at the top, and denser, with few vesicles in the centre portion, and has calcite-filled vertical tubes near the base. The underlying clayey sediment exhibits a prismatic structure at the contact from baking. We cannot yet evaluate whether or not the flow is subaquatic.

Palaeontology

Over 6,000 specimens representing some 40 species of mammals including hominids^{6,7} have been collected from > 250 localities in the Hadar area. They display remarkable preservation due to the unique taphonomic setting noted above. The collection can be closely compared to the Omo 1 Zone^{8,9} (Usno Formation and the Basal Member and Members A and B of the Shungura Formation). Currently this time span in the Omo sequence is dated at ~ 2.6–3.1 Myr (ref. 10). The most significant elements of the assemblage for biostratigraphical correlation include: *Primelephas* (a progressive species with affinities to

Fig. 2 Geological map of northern Hadar.

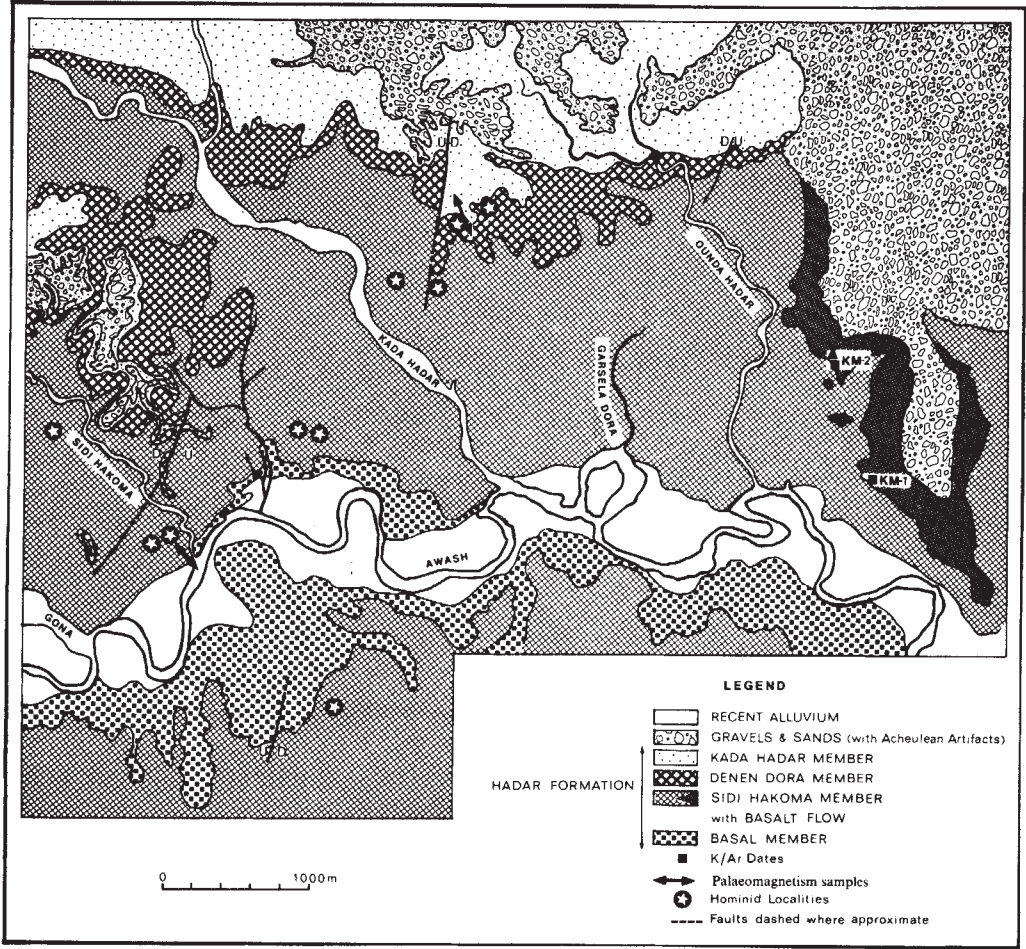
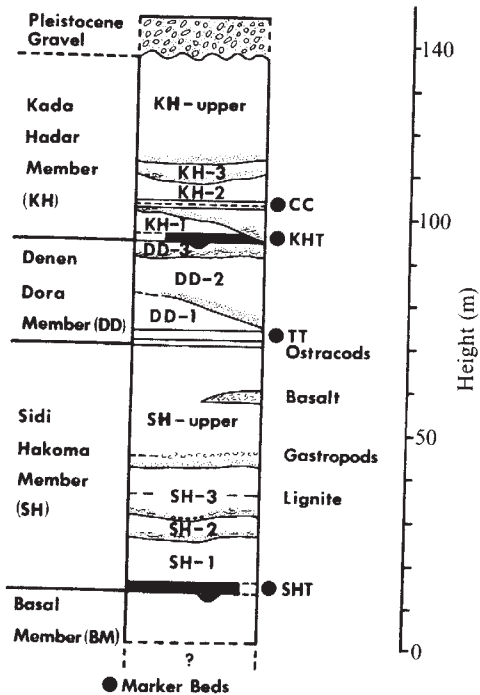


Fig. 3 Geological column of the Hadar Formation.



P. korotorensis from Chad), *Nyanzachoerus pattersoni*, *Notochoerus euilus*, *Loxodonta adaurora*, *Elephas recki* (stages 1 and 2 of Maglio's evolutionary scheme¹¹), *Hipparion* cf. *primigenium* and *Hippopotamus* cf. *imagunculus*. Table 1 presents a provisional faunal list for the Hadar site.

B. T. Gray has suggested a subdivision into three faunal

Fig. 4 Generalised geological cross section of the Hadar Formation. In the east, near Kadada Moumou the upper units have been extended over the basalt for stratigraphic clarity, but virtually all of these have been removed by erosion that predated the Pleistocene gravel.

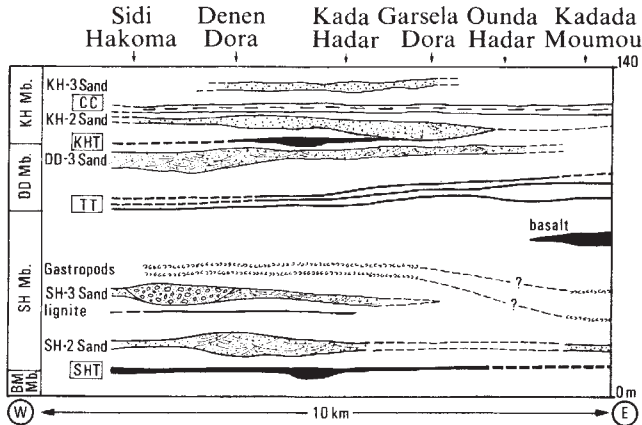


Table 1 Provisional faunal list from Hadar Central Afar

Mammalia	? <i>Elephas</i> sp.	<i>Homo</i> sp.
Artiodactyla	<i>Loxodonta</i> sp. <i>L. cf. adaurora</i>	Chiroptera
Hippopotamidae	<i>Primelephas</i> sp.	gen. et sp. indet.
<i>Hippopotamus</i> sp., <i>H. cf. imagunculus</i>	Deinotherioidea	Aves
<i>Hippopotamus</i> sp.	Deinotheridae	gen. et sp. indet.
Suidae	<i>Deinotherium bozasi</i>	gen. et sp. indet.
<i>Sus</i> sp.	Rodentia	Reptilia
<i>Notochoerus</i> sp., <i>N. cf. euilus</i>	<i>Hystrix</i> sp.	Chelonia
<i>Nyanzachoerus pattersoni</i>	<i>Mastomys</i> sp.	<i>Geochelone</i> sp.
Giraffidae	<i>Xenohystrix</i> sp., <i>X. cf. crassidens</i>	<i>Trionyx</i> sp.
<i>Sivatherium maurusium</i>	<i>Xerus</i> sp.	Crocodylia
<i>Giraffa</i> sp., <i>G. cf. jumae</i>	<i>Tachyoryctes</i> sp.	<i>Crocodylus</i> sp.
<i>Giraffa</i> sp. nov.	<i>Pelomys</i> sp.	Ophidia
Bovidae	<i>Oenomys</i> sp.	gen. et sp. indet.
<i>Ugandax</i> sp.	<i>Tatera</i> sp.	Lacertilia
<i>Tragelaphus</i> sp., <i>T. cf. nakuae</i>	Carnivora	gen. et sp. indet.
<i>Tragelaphini</i> , gen. et sp. indet.	Canidae	Osteichthyes
<i>Hippotragus</i> sp.	gen. et sp. indet.	Siluridae
<i>Kobus</i> sp.	Hyaenidae	gen. et sp. indet.
<i>Aepyceros</i> sp.	<i>Crocuta</i> sp.	Mollusca
aff. <i>Parmularis</i> sp.	Mustelidae	Gastropoda
Alcelaphini, gen. et sp. indet.	? <i>Enhydriodon</i> sp.	gen. et sp. indet.
Neotragini/Cephalophini, gen. et sp. indet.	gen. et sp. indet.	Bivalvia
Ovibovini, gen. et sp. indet.	Felidae	gen. et sp. indet.
Perissodactyla	<i>Megantereon</i> sp.	Arthropoda
Equidae	? <i>Dinofelis</i> sp.	Ostracoda
<i>Hipparion</i> sp., <i>H. cf. primigenium</i>	Primates	gen. et sp. indet.
<i>Hipparion</i> sp.	Cercopithecidae	Decapoda
Rhinocerotidae	<i>Papio</i> sp.	Potamidae
<i>Ceratotherium</i> sp., <i>C. cf. praecox</i>	<i>Theropithecus</i> sp.	gen. et sp. indet.
<i>Diceros</i> sp., <i>D. cf. bicornis</i>	<i>Colobus</i> sp.	
Proboscidea	Hominidae	
Elephantidae	<i>Australopithecus</i> sp., <i>A. aff. robustus</i>	
<i>Elephas recki</i>	<i>Australopithecus</i> sp., <i>A. aff. africanus</i>	

units: lower, middle and upper. The lower unit corresponds to SH-1 and SH-2 submembers, the middle to SH-3 through DD-2, and the upper to DD-3 and above. The vast majority of the middle unit fauna is derived from DD-1 and DD-2. Most taxa are represented in all three units, although proportions do show some interesting variations. The lower unit is characterised by the association of Elephantidae, *Notochoerus euilus*, *Nyanzachoerus pattersoni*, *Tragelaphus cf. nakuae*, *Aepyceros* sp., and cercopithecines. The most common elements of the middle unit are *N. euilus*, *T. cf. nakuae*, *Aepyceros* sp., and abundant *Kobus*. Elephantidae are less well represented. The upper unit contains *N. euilus*, *T. cf. nakuae*, *Kobus* sp., Alcelaphini, *H. cf. primigenium* and Elephantidae.

After more detailed study the subdivision will provide valuable palaeoecological and chronological data. The surprisingly great number of *Kobus* specimens in the middle unit, for instance, or the absence of *Nyanzachoerus* in the upper unit is interesting. Clearly, however, several environmental zones are being sampled in each of these units, and further subdivision is required before detailed interpretations may be proffered.

Geochronological and palaeomagnetic evidence

One of us (J.L.A.) is performing a detailed geochronological study of the Hadar site. Thus far only five K-Ar measurements are available, two for the Kadada Moumou basalt and three for glass shard samples from the SHT channel fill (Fig. 2). The analyses were performed in an extraction system with an ^{38}Ar spike pipette and an MS-10 mass spectrometer with little memory and no mass 36 background. Table 2 presents the analytical data for these samples. Because of the high percentage of atmospheric argon, these determinations are, as yet, relatively imprecise. Two basalt determinations from separate localities in the flow are analytically consistent at 2.95 ± 0.2 Myr and 3.0 ± 0.2 Myr. Both samples consist of fine 0.015 mm plagioclase and augite and minor olivine in a magnetite-rich cryptocrystalline matrix, which comprises 35% of the rock. The content includes 6% of calcite fillings about 0.2 mm in size, surrounded by a fine yellowish, interstitial material (a possible alteration of glass) equalling 1% of the rock's content. The results of the glass shard separates represent three different experimental treatments and are inconsistent, ranging from 3.1–5.3 Myr, each with an analytical uncertainty of 0.3 Myr.

Table 2 Analytical results of K-Ar dating

Sample	$^{40}\text{Ar}^* \text{ g}^{-1}$	$^{40}\text{Ar}^*/^{39}\text{Ar} \times 100$	% $\text{K}_2\text{O}^\dagger$	K-Ar age (Myr) ‡
A Basalt				
KM-1-73	0.0291	20.6	$0.654 \pm 0.004(2)$	3.0 ± 0.2
KM-2-74	0.0327	15.2	$0.748 \pm 0.004(4)$	$2.9_3 \pm 0.2$
B SHT shard§				
4I ₁	0.092	9.0	$1.995 \pm 0.015(2)$	3.1 ± 0.3
4I ₂	0.121	12.3	1.97	4.1 ± 0.3
4I ₃	0.157	16.6	1.98	5.3 ± 0.3
LP6 40/60	$18.14 \pm 0.30(5)$		$10.10 \pm 0.16(10)$	
Biote standard (ref. 1)				

*Radiogenic argon, 10^{-10} mol.

†Average and average deviation, number of measurements in parentheses.

‡ $^{40}\text{K}/\text{total K} = 1.19 \times 10^{-4}$; $\lambda_b = 4.72 \times 10^{-10} \text{ yr}^{-1}$; $\lambda_e = 5.85 \times 10^{-11} \text{ yr}^{-1}$; age uncertainty calculated using 0.3% uncertainty in isotopic composition of sample and atmospheric argon, a 1% uncertainty in argon spike, 1% uncertainty in K_2O , and 1% uncertainty in sample homogeneity.

§Index of refraction = 1.500. All samples 150/200 mesh slide concentrates of flakey bubble sides, 4I₁ and 4I₂ further refined by floating in $\rho = 2.52 \text{ g cm}^{-3}$ liquid; 4I₁ prebaked overnight in vacuum at 250 °C, 4I₂ and 4I₃ at 175 °C; 4I₃ treated 13 min with cold 3% HF.

Until further experiments, these inconsistent results are not understood. They may result from the presence of excess radiogenic argon in the glass. The thicker glass shards, which represent interstices between three or more bubbles, display a microbubble structure which could house such magmatic ^{40}Ar .

Figure 2 shows the location of two sections sampled for preliminary palaeomagnetic examination, in addition to 3 samples of the Kadada Moumou basalt (measurements made by T. J. Schmitt). The lower palaeomagnetic sequence, consisting of 15 samples, spanning 25 m, derives from the basal member up to the SH-2 unit. The upper section comprises 10 samples spanning 20 m of the lower KH member (KH-1, 2 and the lower part of 3). Both sedimentary sequences are magnetised normally, whereas those of the basalt are reversed. All determinations retain their orientations under a.c. demagnetisation. These palaeomagnetic data are consistent with the basalt date, if the reversal is interpreted as representing the Mammoth or Kaena events within the Gauss normal epoch, itself represented by the two normally polarised sedimentary sequences above and below the basalt. This interpretation is to be tested by future palaeomagnetic sample collections from the intervening sedimentary sequence which, to be consistent, should contain the reversal recorded by the basalt.

The Hadar sequence will be a reference from which stratigraphic and palaeontological studies may be extended into adjacent parts of the west central Afar sedimentary basin. The palaeontology, geochronology, and palaeomagnetism

tentatively suggest a short duration of sedimentation for the formation, probably less than a million years with no major time gaps. Thus the Hadar Formation together with its rich fauna and varied geological environments, will permit precise palaeoecological and palaeogeographical reconstruction for an important and previously poorly known segment of later Cainozoic hominid evolution.

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Plio–Pleistocene hominid discoveries in Hadar, Ethiopia

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The International Afar Research Expedition has now recovered remains of twelve hominid individuals from geological deposits estimated to be ~ 3.0 Myr in Hadar, Ethiopia. A partial skeleton represents the most complete hominid known from this period. The collection suggests that Homo and Australopithecus coexisted as early as 3.0 Myr ago.

FOLLOWING a short reconnaissance expedition in 1972 (ref. 1) to the central Afar, one of us (M.T.) organised the International Afar Research Expedition. We have now codirected two field campaigns (September–December, 1973 and 1974 (refs 2 and 3)) in the area known as Hadar (see Fig. 1).

Although a number of other excellent fossil sites are known from the central Afar⁴, Hadar was selected for intensive exploration for a number of reasons: first, the fossil vertebrate assemblage suggested a considerable antiquity (~ 3–4 Myr); second, the area is extremely rich in splendidly preserved fossils, deposited in low energy environments; third, the deposits are heavily dissected and characterised by clear marker-horizons which are laterally continuous; and fourth, the thick series of lake sediments contains a number of volcanic horizons available for absolute radiometric age determinations.

The Hadar site is located in the Afar depression in the west central Afar sedimentary basin⁵ at 11°N and 40°30'E. It now encompasses ~ 42 km², but extension of study into

adjacent, previously unexplored regions will substantially increase the area.

Preliminary palaeontological investigations, particularly of the suids and the elephants, have suggested a biostratigraphic correlation of the Hadar Formation with the Usno and the lower portion of the Shungura Formations^{6,7}. Two radiometric K–Ar age determinations for a basalt support this with an age estimate of 3.0 ± 0.2 Myr (ref. 5).

All hominid fossils (Table 1), and most of the vertebrate material, were surface finds, although some small scale excavation and sieving operations were undertaken. A painted marker was placed at each palaeontological locality, each located on a map and the geological section carefully examined to determine the horizon yielding fossil material (see Fig. 2). At each hominid locality, a topographical map and detailed stratigraphical sections were drawn. Samples of matrix adhering to fossil hominid specimens are undergoing mineralogical and sedimentological analyses for comparison with sediment samples taken at their collection points. Their completeness and lack of anataxic abrasion⁷ virtually precludes the possibility that the specimens were moved over great distances.

1973 hominid discoveries

The first discovery of fossil hominid remains in the Hadar region occurred on October 30, 1973, when four associated leg bone fragments (AL 128, 129) were collected from a mudstone horizon. These consist of fragmentary right and left proximal femora associated with a right proximal tibia

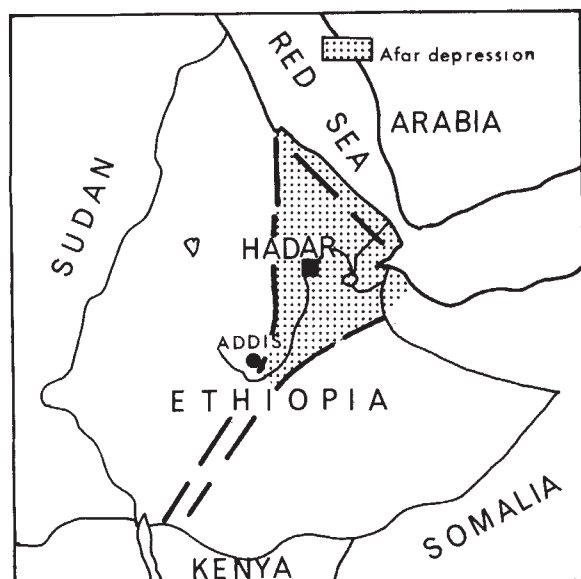


Fig. 1 Location of Hadar.

and distal femur. Their proximity to one another, as well as the morphological and size similarities of the proximal femora, strongly suggest that they represent a single individual. This provides a complete knee which will greatly enhance our understanding of the biomechanics of this important joint in early hominids (see Fig. 3).

Both proximal femora lack heads and necks. The left is best preserved with the shaft broken ~ 38.0 mm below the lesser trochanter. From the remaining portions of the femora it is clear that the neck is flattened anteroposteriorly and that its cross section is oval. There is an indication of trochanteric flare, the trochanteric fossa is well marked, a quadrate tubercle is clearly discernible, muscle markings are prominent on the greater trochanter, the spiral line is pronounced and the lesser trochanter is visible from the anterior aspect. Both fragments, particularly the right, exhibit some degree of predepositional crushing, which is

suggestive of carnivore activity.

The distal femur is undistorted, and both condyles are complete and intact. It is small, with the lateral condyle measuring 39.0 mm anteroposteriorly and 18.9 mm mediolaterally. This fragment demonstrates a number of anatomical details which are intimately related to bipedal locomotion^{8,9}: the bicondylar angle is rather high, the lateral lip of the patellar groove is raised and the lateral condyle flattened and elongated.

The associated proximal tibia is intact except for some slight abrasion around the periphery of the superior articular surface. It also is small, the total preserved articular surface measuring 50.7 mm mediolaterally and 33.0 mm

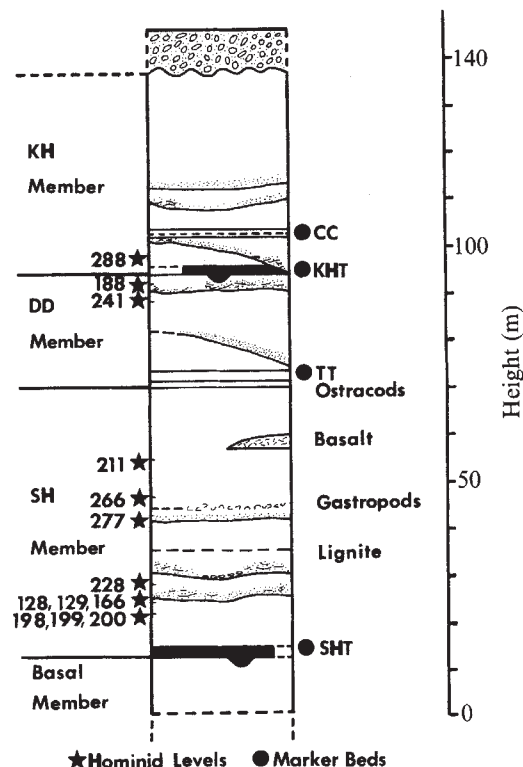


Fig. 2 Stratigraphic positions of Hominid Horizons. ★, Hominid levels; ●, marker beds.

Table 1 Fossil hominid specimens from Hadar

Afar locality (AL)	Date of discovery	Description
1973		
128-1	30 October	Left proximal femur fragment
129-1a	30 October	Right distal femur
129-1b	30 October	Right proximal tibia
129-1c	30 October	Right proximal femur fragment
166-9	11 December	Left temporal fragment
1974		
188-1	16 October	Right mandibular corpus; M ₂ -M ₃
198-1	18 October	Left mandibular corpus; C-P ₄ , dm, M ₁ -M ₂
198-17a,b	5 December	Left I ¹ and I ²
198-18	5 December	Right I ₂
199-1	17 October	Right maxilla; C-M ³
200-1a,b	17 October	Complete maxilla; 16 teeth. Right M ₁
211-1	20 October	Right proximal femur fragment
228-1	27 October	Diaphysis of right femur
241-14	22 December	Left lower molar
266-1	16 November	Mandibular corpus; left P ₃ -M ₁ , right P ₃ -M ₃
277-1	19 November	Left mandibular corpus; C-M ₂
288-1	24 November	Partial skeleton: occipital and parietal fragments; mandibular corpus with left P ₃ , M ₃ , right P ₃ -M ₃ , mandibular condyles; right scapula fragment; right humerus; proximal and distal left humerus; proximal and distal right and left ulnae; proximal and distal right radius; distal left radius; left capitate; 2 phalanges; 6 thoracic vertebrae and fragments; 1 lumbar vertebra; sacrum; left innominate; left femur; proximal and distal right tibia; right talus right distal fibula; numerous rib fragments.

anteroposteriorly. The tibial tuberosity is pronounced and is limited proximally by the transverse groove; the soleal line is distinct on the posterior surface of the shaft and the interosseous membrane attachment is indicated by a roughened line on the medial aspect of the shaft; the head is slightly retroflexed with only minor tibial torsion; and the intercondyloid eminence is prominent with well developed intercondyloid fossae.

A heavily eroded temporal fragment (AL 166-9) constitutes the only other hominid discovery from the 1973 field season. The specimen derives from a sandstone horizon a few metres stratigraphically above the level of the postcranial material. The temporal has been extensively eroded, with most of the petrous portion broken away, and extensive pneumatization is exposed in the mastoid and zygomatic regions. The mandibular fossa is broad and flat, bordered by a postglenoid process, but open anteriorly with only a slight articular tubercle. There is a prominent entoglenoid process on the medial wall which is broken exposing a large pneumatization. The mastoid is large and globular. The external auditory meatus is oval in section with a thick tympanic plate.

1974 hominid discoveries

Our sample of the Hominidae has been greatly augmented with additional field work. The 1974 investigations yielded



Fig. 3 Anterior view of AL 129-1a and 1c

remains of 10 additional individuals represented by dental, cranial and postcranial elements, including a remarkably intact partial skeleton.

Stratigraphically just below the 1973 hominid discoveries, one complete (AL 200-1) and one half maxilla (AL 199-1) (see Fig. 4) were recovered by Ato Alemayehu Asfaw within 15 m of one another and from the same mudstone horizon.

The AL 200 specimen is an undistorted palate with full dentition and an associated right M_1 . The general pattern of dental wear is interesting, in that the incisors exhibit extensive 'ribbon like' wear and dentine exposure relative to the postcanine teeth. In addition, the canines and the P^3 s have suffered antemortem enamel chippage on their buccal surfaces during the specimen's lifetime. The tooth rows of AL 200 are subparallel and the arch is relatively long. The anterior portion of the arch is broad to accommodate the large central incisors and marked diastemata occur between the lateral incisors and the canines. The palate is shallow, becoming somewhat deeper posteriorly.

In lateral view this specimen exhibits pronounced alveolar prognathism. The zygomatic root is situated above the first molar, the maxillary sinuses are large and the lower nasal margin is guttered.

The right half maxilla is smaller in size and is less complete, with $C-M^3$, as well as both incisor sockets and the

I^2 root. The dentition is smaller than that of AL 200 (Table 2), but the similarity in morphological detail is striking. Although the incisors are lacking, it is apparent from the root sockets that the anterior portion of the arch was broad and somewhat squared-off as in AL 200. The specimen is broken close to the midline exposing the incisive canal. The palate is shallow and the greater palatine foramen is present. The inferior nasal margin is not sharp and in lateral view alveolar prognathism is well developed. The maxillary sinus is large and the zygomatic root is situated above the distal portion of the first molar.

A partial mandible (AL 266-1) was located in a horizon of sandy clay, ~ 10 m stratigraphically below the basalt flow³. This specimen includes the complete right corpus with P_3-M_3 , the symphysis with canine and incisor roots, and a portion of the left corpus with P_3-M_1 (Table 3). The dentition is moderately worn with the third molar having just come into occlusion and showing only minor wear facets. The anterior portion of the dental arch is rounded and the dental rows are straight and slightly divergent posteriorly. The post-incisive planum is only moderately developed and the corpus in the region of M_1 is 21.7 mm thick and 31.0 mm deep.

A right mandibular fragment (AL 188-1) containing M_2-M_3 and roots of P_3-M_1 , was collected from a sandstone horizon immediately below KHT. The molars are heavily worn. The mandible exhibits some wind abrasion, but it is possible to estimate a thickness of 20.7 mm and a depth of ~ 32.0 mm in the region of M_1 .

AL 277-1, a fragmentary left mandible containing $C-M_2$ and sockets for I_1-I_2 , is derived from a sandstone horizon. It is broken just to the right of the midline and distal to M_2 . The occlusal wear is heavy with the canine worn flat. Anteriorly the fragment gives the impression of being deep, measuring ~ 41.0 mm just distal to P_3 . A prominent post-incisive planum is present, as well as a slight inferior mandibular torus. The inferior surface of the symphysis is flattened and has distinct mental spines. The corpus exhibits slight eversion.

An interesting left half mandible (AL 198-1) was recovered from a mudstone horizon, stratigraphically equivalent to AL 199 and AL 200. The specimen is broken near the midline with the I_1 socket and the root for I_2 preserved. The $C-P_4$ and M_1-M_2 are present, with a retained deciduous molar located distal to the P_4 . The deciduous nature of this tooth is confirmed by a radiograph demonstrating a widely divergent root system. Substantial occlusal wear is evident on P_4-M_2 . The corpus is broken just distal to M_2 , exposing a portion of the mesial root of M_3 immediately superior to the mandibular canal. The mandible is lightly built, being 16.0 mm thick and 31.9 mm deep at M_1 .

Fig. 4 Occlusal views of AL 200-1a and AL 199-1

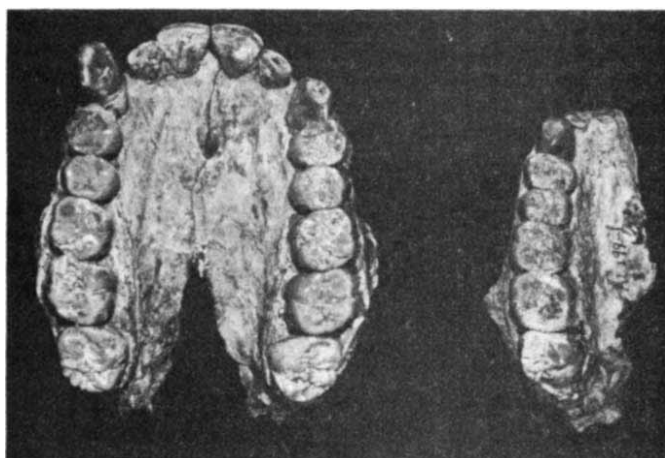


Table 2 Dental measurements of AL 200-1a and AL 199-1 (mm)

	Left		Right	
	Mesio-distal	Bucco-lingual	Mesio-distal	Bucco-lingual
AL 200-1a				
I^1	10.8	8.3	10.9	8.5
I^2	7.4	7.1	7.3	7.0
C	9.4	10.9	9.4	11.0
P^3	9.0	12.2	8.9	12.2
P^4	8.5	12.2	8.5	12.1
M^1	11.8	13.1	11.8	13.2
M^2	13.8	14.8	13.7	15.0
M^3	14.2	15.0	14.3	15.0
AL 199-1				
C			8.7	9.3
P^3			7.3	11.2
P^4			7.1	11.2
M^1			10.1	12.0
M^2			11.7	13.5
M^3			11.3	(12.7)

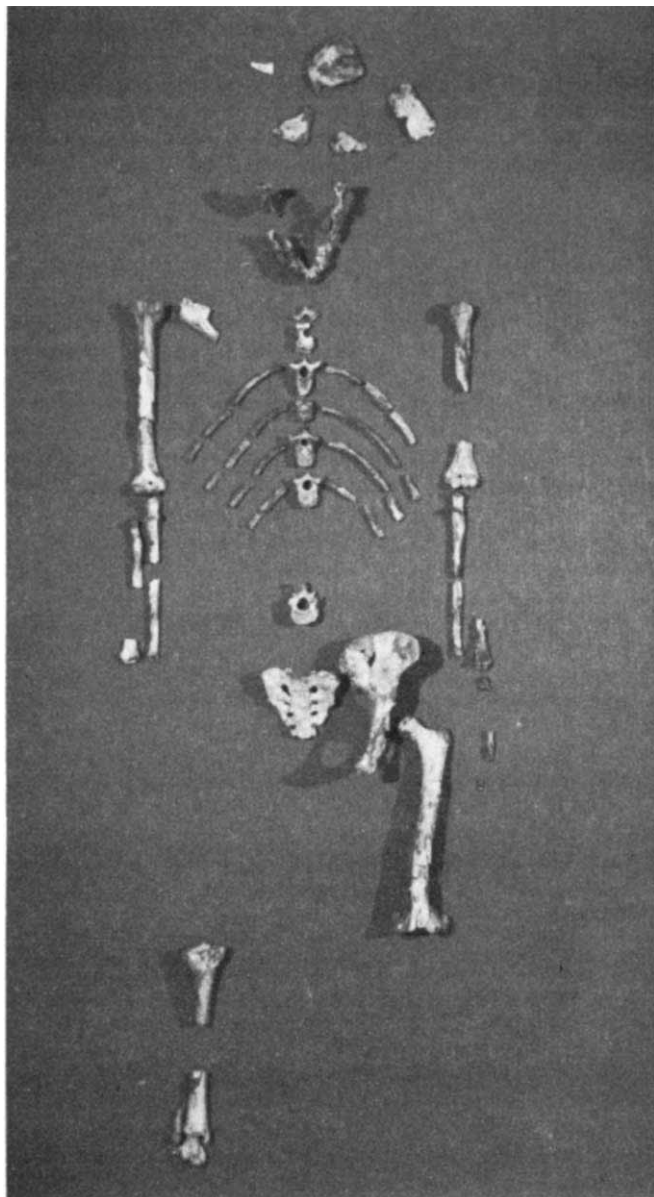


Fig. 5 Partial skeleton (AL 288-1) from Hadar

It exhibits some modelling and the inferior margin is thin and not bulbous.

During screening operations, three additional teeth were located at this locality: a right I_2 (AL 198-18), a left I^2 (AL 198-17a) and a left I^1 (AL 198-17b). The interproximal facets on the upper incisors match perfectly, suggesting that they are from the same individual. Because of the close association of these teeth with the AL 198 mandible, we tentatively assign them to the same individual.

Stratigraphically just below the basalt flow a fragmentary right proximal femur (AL 211-1) was collected. The specimen is somewhat abraded with the head and neck missing and most of the greater trochanter. The neck is very flattened, no trochanteric flare is present, the intertrochanteric line is weakly expressed, the spiral line is well developed and the lesser trochanter is not visible from the anterior aspect. The specimen is quite large and fairly robust.

AL 228-1 is a distal portion of a femoral diaphysis recovered from a sandstone. It is not large, but exhibits strong development of the linea aspera. It is smaller than AL 129-1A.

The partial skeleton

The discovery on November 24 of a partial skeleton (AL 288-1; see Fig. 5) eroding from sand represents the most

outstanding hominid specimen collected during the 1974 field season. The stratigraphic horizon yielding the skeleton is situated just above the KHT, which has not yet been dated. Fossil preservation at this locality is excellent, remains of delicate items such as crocodile and turtle eggs and crab claws being found. It is obvious that this discovery provides us with a unique opportunity for reconstructing the anatomy of an early hominid in far more detail than has been previously possible. Extensive descriptive and comparative studies are projected for the AL 288 partial skeleton and will provide us with details of stature, limb proportions, articulations and biomechanical aspects. Three weeks were devoted to intensive collecting and screening to ensure the recovery of all bone fragments from the site. Laboratory preparation and analysis has only just begun, and in this report it is possible to mention only a few salient points.

The mandible is not heavily built; it is 30.0 mm deep and 19.0 mm thick in the region of M_1 . The M_3 s are fully erupted and occlusal wear facets are just appearing. The symphysis is intact with a slight post-incisive planum. Although the incisor crowns are absent, it is apparent that this region was quite small. The remaining dentition is small and not very worn. The P_3 s are interesting with a sloping buccal surface and almost no development of a metaconid. The form of the dental arch as well as the body of the mandible is distinctly V-shaped.

The cranium is not sufficiently complete to estimate cranial capacity. The cranial bones are thin, exhibit no sutures (internally or externally) and no marked development of nuchal or temporal musculature is present.

The left innominate is complete, although it is somewhat distorted in the pubic region and particularly in the area of sacral articulation. In size the specimen resembles Sterkfontein (Sts) 14; the ilium, however, gives the appearance of being higher, and the anterior border is relatively straight. A strongly developed anterior inferior spine is apparent. The acetabulum is shallow when compared with modern man and with Sts 14. The sciatic notch is broad, the subpubic angle obtuse and the pubis exhibits a pronounced ventral arc, all of which suggests the skeleton belonged to a female. When viewed from the superior aspect, the base of the sacrum is divided into thirds, with the diameter of the sacral body equal to each of the alae. This again suggests that AL 288 was female.

A complete left femur is associated with the innominate, but the distal portion is badly crushed. Its total length has been estimated at 280 mm but may be slightly revised when the distal end is reconstructed. The femur has minimal trochanteric flare, the neck is anteroposteriorly flattened, an intertrochanteric line is present and the lesser trochanter is not visible from the anterior aspect.

The proximal right tibia is nearly identical in size and morphology to AL 129-1b. The distal tibia is associated, and articulates with a talus and a distal fibula.

The right humerus is complete with some crushing of the proximal end. Its total length is estimated at ~ 235 mm giving a value of 83.9 for the humeral-femoral index. The distal end possesses a marked ridge separating the capitulum and trochlea.

Significance of the Hadar hominids

Detailed studies of the Hadar hominids have just recently been initiated and definitive interpretations are not yet possible. Because of the geological antiquity, and in some instances the completeness of the specimens, however, we proffer a number of preliminary impressions concerning their phyletic affinities and resemblances to other specimens.

The partial skeleton exhibits a number of similarities to the Sterkfontein sample. Specifically, the size of the pelvis and to some degree its morphology are reminiscent of Sts 14. The shallow acetabulum and relatively high ilium, how-

Table 3 Dental measurements of AL 266-1(mm)

AL 266-1	Left		Right	
	Mesio-distal	Bucco-lingual	Mesio-distal	Bucco-lingual
P ₃	9.1	10.1	9.2	10.1
P ₄	8.9	11.0	9.4	10.8
M ₁	12.1	11.9	12.1	12.0
M ₂			13.3	14.0
M ₃			15.3	13.7

ever, demonstrate certain divergences from the Sterkfontein specimen and possibly reflect a somewhat more primitive status for the AL 288 specimen. The associated V-shaped mandible is noteworthy. Leakey¹⁰ has drawn attention to mandibular shape and has suggested that KNM-ER 1482 (Kenya National Museum, East Rudolph), and Omo 18 should be considered primitive because of their V-shaped contours. Should this prove to be a diagnostic character, it is possible that AL 288 retains more primitive features than *Australopithecus africanus*, recognised from Sterkfontein.

Previously the 1973 postcranial material has not been assigned to a taxon. It is now clear that it should probably be included in the same category as the AL 288 specimen because of the striking similarity of the proximal tibial fragments in size and morphology as well as the preserved femoral fragments. This is important because: there is now evidence of at least two individuals of a very small hominid in Hadar, and the AL 128 and 129 specimens are situated stratigraphically 80 m below the partial skeleton (Fig. 2).

The presence of another taxon is suggested by two other specimens in the Hadar hominid collection. AL 211-1 resembles very closely Olduvai Hominid (OH) 20 (ref. 11) as well as the two proximal femora from Swartkrans (SK 82 and SK 97). Similarities are not only in size but also in morphological detail; stout shafts, flattened necks, a lack of trochanteric flare and posteriorly facing lesser trochanters not visible in anterior view.

The temporal fragment (AL 166-9) resembles material from Swartkrans and East Rudolf which has also been assigned to a large *Australopithecus* pattern, particularly in the large bulbous mastoid process and heavy pneumatization, as well as the broad temporal shelf. It is, however, less typical of this pattern in the broad, flat mandibular fossa and absence of a strong articular tubercle.

The close association of AL 199-1 and AL 200-1, as well as their stratigraphic equivalence, is important because

of the high probability that they sample the same taxon. Except for size differences, these two specimens are remarkably similar, and suggest variation within a single taxon. The complete maxilla has large canines, broad central incisors and large posterior dentition. These characters as well as other details suggest resemblances with some *Homo erectus* material, particularly *Pithecanthropus* IV. This dental pattern is also seen in KNM-ER 1590 from East Rudolf, a specimen with large cranial capacity assigned to the genus *Homo*^{10,12}. It must be recognised that other aspects of the AL 200 maxilla are 'primitive', such as the guttered nasal margin and the alveolar prognathism.

The AL 266-1 and AL 277-1 mandibles resemble, in details of the dentition and mandibular morphology, other specimens assigned to *Homo*, such as OH 7 (ref. 13) and KNM-ER 1802 (ref. 10).

On the basis of the present hominid collection from Hadar it is tentatively suggested that some specimens show affinities with *A. robustus*, some with *A. africanus* (*sensu stricto*), and others with fossils previously referred to *Homo*.

The understanding of Plio-Pleistocene hominid remains is undergoing intensive revision and re-evaluation, and we stress that our identifications and phylogenetic interpretations such as the guttered nasal margin and the alveolar prognathism. With continued collection of specimens from the three million year-old time range in Hadar and elsewhere, and with additional detailed studies and comparisons, our attempts to interpret the earliest hominids should become more clear.

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Sequence of promoter for coat protein gene of bacteriophage fd

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The nucleotide sequence in the promoter region for the coat protein gene of phage fd has been determined. This sequence contains an endonuclease R·Hha cleavage site at the fifteenth nucleotide upstream from the RNA start site. Cleavage results in loss of promoter function. Comparison with the sequence of another fd promoter indicates that the longest sequence common to both was TATAAT in the region in which RNA polymerase forms a stable initiation complex.

ELUCIDATION of the structure of DNA specifying the initiation of transcription is essential to understand the mechanism of gene expression. Although the sequences of several different promoters have been determined¹⁻⁷, the information still seems to be insufficient to define the structure essential for promoter function. In previous work in which the promoter regions were localised on the cleavage map of fd^{8,9}, two strong promoters were shown to be located on two separate fragments, named *HapC* and *Hap-HgaV*. The nature of these promoters was very similar: RNA polymerase binds tightly to these fragments in the presence of GTP and initiates G-start RNA with almost the same efficiency. The sequence in the promoter

region on *Hap-HgaV* (named G2 promoter) has been determined⁷. In this report, the promoter region on *HapC* (named G3 promoter) was sequenced to find the sequence common to both promoter regions.

HapC contains the entire gene for the coat protein (B protein) of fd^{10,11}, and RNA of about 350 bases (G3 RNA) is efficiently synthesised on this fragment. This RNA contains the sequence for the coat protein (M.T. *et al.*, unpublished). Restriction endonuclease (R) *Hae*III cleaves *HapC* into two pieces: *HapC-Hae 1* and *HapC-Hae 2*. RNA of about 200 bases (G3' RNA) is synthesised only on *HapC-Hae 1*. Comparison of the sequences of G3 and G3' RNAs indicated that G3' RNA was yielded by termination of G3 RNA at the R·*Hae*III cleavage site. *HapC-Hae 1* can be further cleaved by R·*Hha* into two pieces, *Hha 1* and *Hha 2*. No RNA was initiated, however, on the resulting fragments; the ability to form a stable complex with RNA polymerase was also destroyed by this cleavage. The positions of the cleavage sites and of the G3 RNA start site on *HapC* are indicated in Fig. 1. The sequences in the region preceding the G3 RNA start site and in the region in which RNA polymerase forms a stable initiation complex (RNA polymerase binding site) were determined by the following procedures.

(1) In low salt conditions, *HapC-Hae 1*-directed RNA synthesis was initiated at two additional sites, although RNA initiation at the G3 promoter was still predominant. Figure 2a

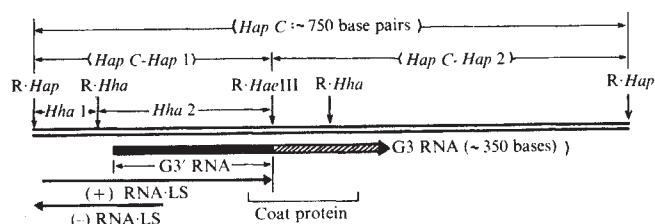


Fig. 1 Cleavage and transcription maps of the *HapC* region of fd DNA. Location of *HapC* on the cleavage map of fd DNA is given in ref. 8.

and *b* shows autoradiographs of the low salt and high salt (0.15 M KCl) transcripts for comparison. The direction of transcription and the regions covered by these transcripts have been determined by hybridisation to fd phage (+ strand) DNA and each fragment of *HapC*. Since both the low salt transcripts, named (+)RNA·LS and (-)RNA·LS, covered the region preceding the G3 RNA start site (Fig. 1), these RNAs were further fractionated by hybridisation to each *Hha* fragment and by partial T1 RNase digestion, and the sequences of the resulting fragments were determined.

(2) *HapC-Hae 1* forms a stable initiation complex with RNA polymerase in the presence of GTP: the region protected by RNA polymerase was isolated after treatment with DNase I. In accordance with other observations^{6,12-14}, the isolated fragment was about 45 base pairs, as determined by gel electrophoresis. The polymerase-protected fragment was denatured by heating, and incubated in an RNA-synthesising system containing a primer oligonucleotide¹². The resulting transcripts were fractionated by hybridisation to fd phage DNA and by gel electrophoresis, and their sequences determined.

The procedures for preparation of fd replicative form DNA (rfDNA) and *Escherichia coli* RNA polymerase holoenzyme, and the conditions used for digestion of DNA and gel electrophoresis have been described previously^{8,9,12}. For preparation of *HapC-Hae 1*, *HapC* was first isolated from the R·*Hap* digest of fd rfDNA, redigested with R·*Hae*III, and then fractionated by gel electrophoresis. R·*Hap*, R·*Hae*III and R·*Hha* were respectively prepared from *Haemophilus aphrophilus*, *H. aegyptium* and *H. haemolyticus* by the procedures described previously¹⁵. For sequencing, RNA was synthesised

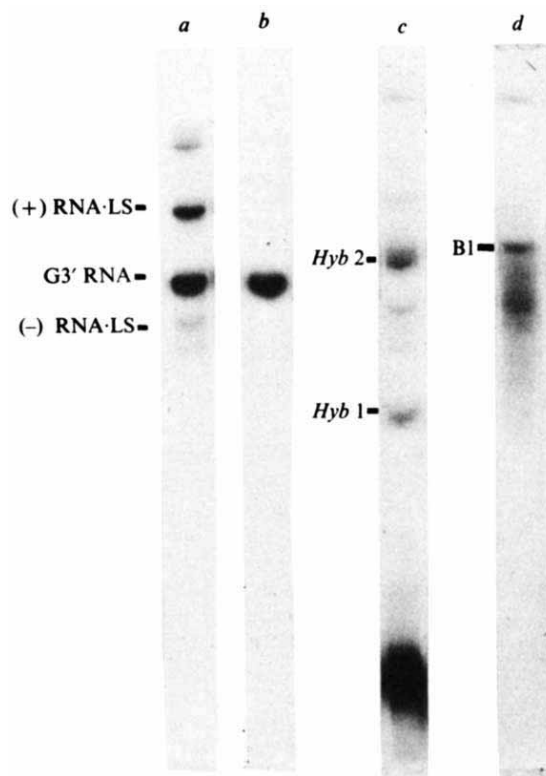


Fig. 2 Gel electrophoretic patterns of *a*, RNA formed on *HapC-Hae 1*, at low salt; *b*, RNA formed on *HapC-Hae 1* at high salt; *c*, portions of (+)RNA·LS hybridised to the R·*Hha*-digest of *HapC-Hae 1*, and *d*, RNA formed on the RNA polymerase-protected fragment. *a*, *HapC-Hae 1* (about 2 pmol) was incubated for 1 h at 37 °C in a reaction mixture (1 ml) containing 8 mM MgCl₂, 40 mM Tris (pH 7.9), 0.1 mM dithiothreitol, 50 μM (α³²P)GTP and 0.2 mM each of three other NTPs, and about 15 pmol RNA polymerase. The transcripts were isolated and electrophoresed for 3 h at 150 V on a 5% polyacrylamide gel (0.6 cm × 12 cm) containing 7 M urea, as in ref. 12. *b*, RNA was synthesised on *HapC-Hae 1* and electrophoresed as in (*a*), except that 0.15 M KCl was added to the reaction mixture. *c*, RNA was extracted from the (+)RNA·LS band of (*a*) and mixed with about 10 pmol of the R·*Hha*-digest of *HapC-Hae 1* in 0.6 M NaCl-0.06 M Na citrate. The mixture was heated for 5 min at 100 °C and then held for 3 h at 55 °C. After cooling, the mixture was diluted, treated with 100 units of T1 RNase for 1 h at 37 °C, and electrophoresed for 3 h at 150 V on a 10% gel¹². *d*, *HapC-Hae 1* (about 10 pmol) was mixed with about 30 pmol of RNA polymerase in a binding mixture (1 ml) containing 8 mM MgCl₂, 40 mM Tris (pH 7.9), 0.15 M KCl, and 0.1 mM GTP, and the portion protected from DNase digestion was isolated after treatment with DNase I, as in ref. 12. The isolated fragment was heated for 5 min at 100 °C and added to a reaction mixture (1 ml) containing 8 mM MgCl₂, 50 mM KCl, 40 mM Tris (pH 7.9), 0.1 mM dithiothreitol, 20 μM each (α³²P)GTP and the other NTPs, 10 nmol AAAC and about 10 pmol RNA polymerase. After incubation for 3 h at 37 °C, the transcripts were isolated and mixed with 50 μg of fd phage DNA in 0.9 M NaCl-0.09 M Na citrate. The mixture was heated for 5 min at 100 °C and then held for 3 h at 51 °C. The fraction hybridised to fd phage DNA was isolated by using a Millipore filter and electrophoresed for 3 h at 150 V on a 15% gel containing 7 M urea, as in ref. 12.

with each of four (α³²P) nucleoside triphosphates (NTP), and hydrolysed with T1 RNase. The resulting nucleotides were fractionated by two-dimensional chromatography on a polyethyleneimine (PEI)-cellulose plate (Macherey-Nagel)¹⁶. Distribution of ³²P in spots was determined to estimate molar yields. To analyse the sequences of oligonucleotides, each spot was digested with either pancreatic RNase or U2 RNase and chromatographed using a two-dimensional system. The nucleotides produced were further digested with T2 RNase, and the distribution of ³²P in nucleotides determined. The starting nucleotides were not determined, since the nucleotides con-

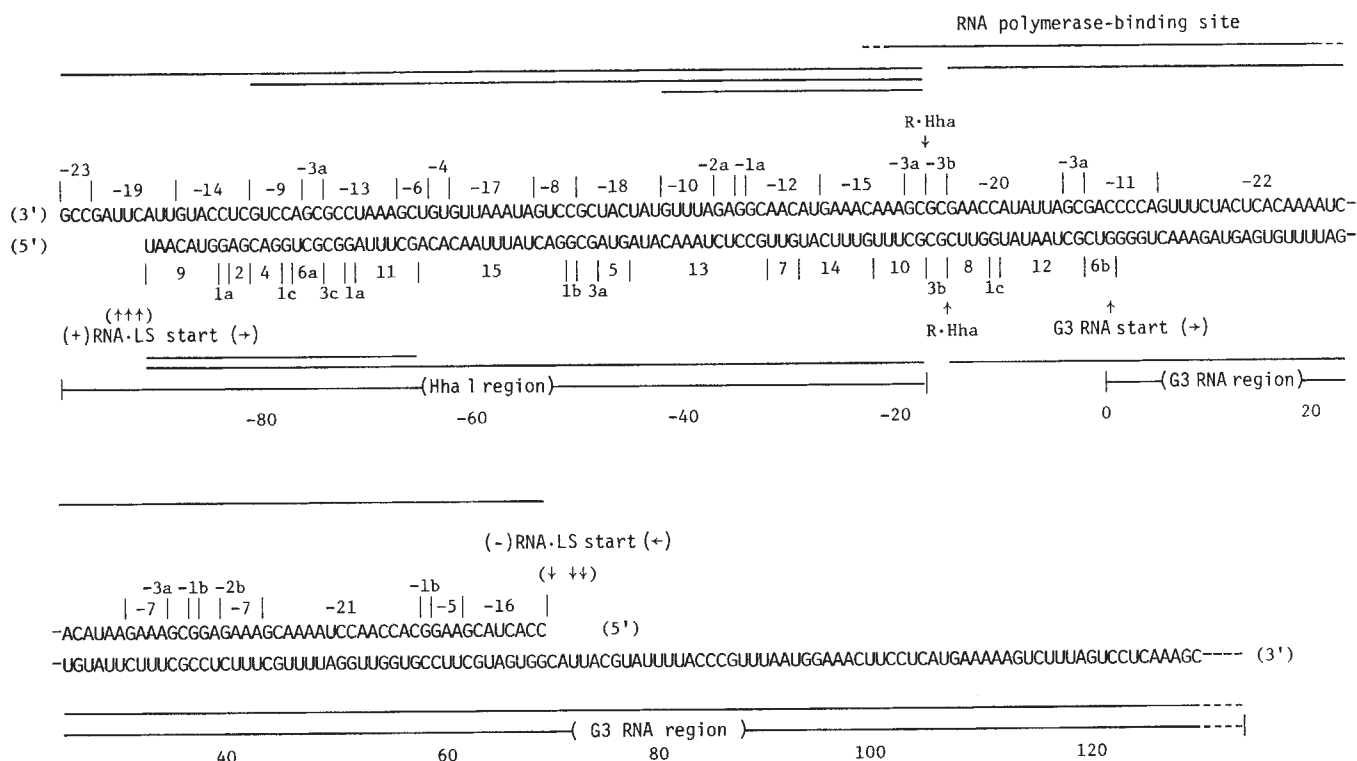


Fig. 3 Arrangement of T1 RNase nucleotides produced from the low salt transcripts of *HapC-Hae I*. Nucleotide numbers correspond to those given in Table 1. As the starting nucleotides were not identified, the possible start sites of (+)RNA·LS and (-)RNA·LS are indicated in parentheses. Sites corresponding to the R·Hha cleavage sites are indicated by arrows.

taining triphosphate ends stayed at the origin in the conditions used for chromatography.

Sequence in region preceding G3' RNA start site

The nucleotides produced from G3' RNA by digestion with pancreatic and T1 RNases have been analysed, and the sequence of 130 nucleotides from the 5' end of G3' RNA has been determined by analysis of its partial T1 RNase digest (Fig. 3 'G3 RNA region', our unpublished observation). The sequence contains the ribosome binding site number 1 of phage f1 messenger RNA¹⁷ in the positions 96–120. Figure 4a and b shows fingerprints of T1 RNase nucleotides produced from G3' RNA and (+)RNA·LS for comparison. By comparing the sequences of the products, (+)RNA·LS was found to contain 22 'extra'

nucleotides. The positions of the extra nucleotides and their sequences are shown in Fig. 4b and Table 1a. These nucleotides were assigned to the region preceding the G3 RNA start site. To arrange these nucleotides, (+)RNA·LS was hybridised to the R·Hha digest of *HapC-Hae I*. The regions protected by hybridisation to the DNA fragments, *Hha 1* and *Hha 2*, were isolated by gel electrophoresis after treatment with T1 RNase (Fig. 2c). The *Hha 1* and *Hha 2* regions were digested with T1 RNase and fractionated using a two-dimensional system. Among the 22 T1 RNase nucleotides of (+)RNA·LS assigned to the left of the G3 RNA region, 17 nucleotides arose from the *Hha 1* region, as indicated in Fig. 4c, and the remaining nucleotides from the *Hha 2* region. The order of the T1 RNase nucleotides in the *Hha 1* region was determined by analysis of its partial T1 RNase digests and of the T1 RNase nucleotides

Table 1 T1 RNase nucleotides produced from the low salt transcripts of *HapC-Hae I*

a, Nucleotides from (+)RNA·LS

T1a: G A (2), T1b: G C (1), T1c: G U (2), T2: AG C (1), T3a: CG A (1), T3b: CG C (1), T3c: CG G (1), T4: CAG G (1), T5: AUG A (1), T6a: UCG C (1), T6b: CUG G (1), T7: UUG U (1), T8: CUUG G (1), T9: UAACAUG G (1), T10: UUUCG C (1), T11: AUUUCG A (1), T12: UAUAUUCG C (1), T13: (AU,AC)AAAUC(C,U)CG U (1), T14: UACUUUG U (1), T15: (AC,AC,AAUUU,AUC)AG G (1).

b, Nucleotides from (-)RNA·LS

-T1a: G A (1), -T1b: G C (2), -T2a: AG A (1), -T2b: AG G (1), -T3a: CG A (4), -T3b: CG C (1), -T4: UG U (1), -T5: AAG G (1), -T6: UCG A (1), -T7: AAAG A (2), -T8: CCUG A (1), -T9: ACCUG C (1), -T10: AUUUG U (1), -T11: ACCCCAG C (1), -T12: UACAACG G (1), -T13: AAAUCCG C (1), -T14: C(C,U)CAUG U (1), -T15: AAACAAAG U (1), -T16: CCACUACG A (1), -T17: AUAAAUUG U (1), -T18: UAUCAUCG C (1), -T19: UUACUUAG C (1), -T20: (AUU,AU,ACC)AAG C (1), -T21: C(ACC,AACC,U)AAAACG A (1), -T22*: (A₁₀,C₈,U₈)UG A (1), -T23: CCG (>0.5)

*Pancreatic RNase digestion yields CA, CU, UA, UC, 2UU, ACA, 2ACU, AUC, AAUA, and AAAACA.

In a, nucleotides assigned to the region preceding the G3 RNA start site are listed. Nucleotide numbers correspond to those indicated in Fig. 4. Nucleotides produced from (-)RNA·LS are denoted by the minus sign. Nucleotides linked to the 5' ends are underlined. Molar yields are indicated in parentheses.

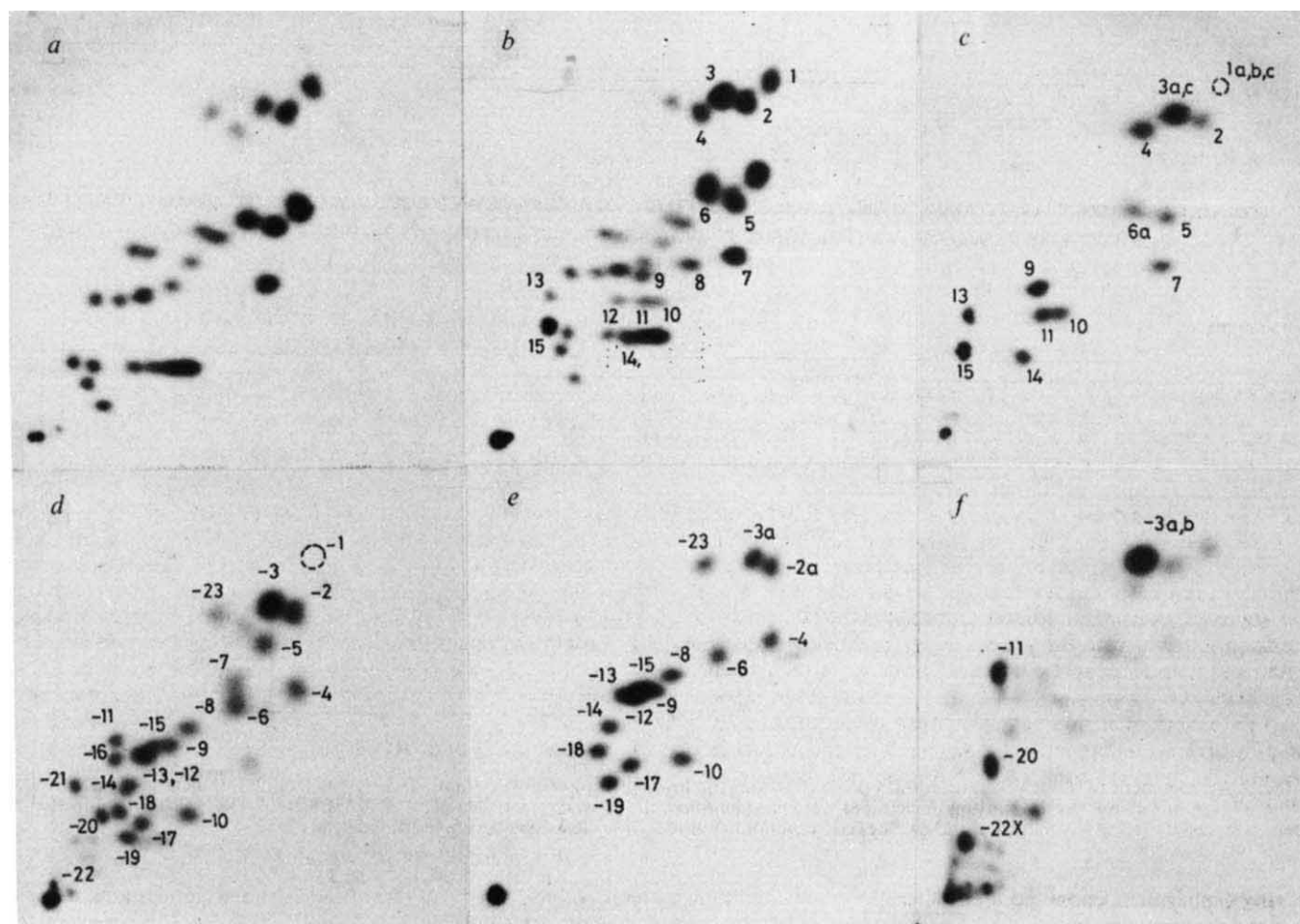
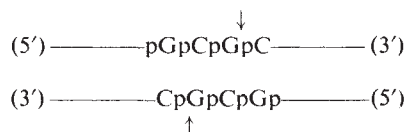


Fig. 4 Two-dimensional fingerprints of T1 RNase digests of *a*, G3' RNA; *b*, (+)RNA·LS; *c*, portion of (+)RNA·LS hybridised to the DNA fragment *Hha* I (Fig. 2c, *Hyb* I region); *d*, (-)RNA·LS; *e*, portion of (-)RNA·LS hybridised to the DNA fragment *Hha* I; and *f*, RNA formed on the RNA polymerase-protected fragment (Fig. 2d, B1 region). All RNAs have been synthesised with α - ^{32}P -GTP, and the digests chromatographed on 20 $\times$ 20 cm PEI-cellulose plates, as in ref. 16. The sequences of the spots numbered are given in Table 1.

produced from the corresponding region of (-)RNA·LS (Fig. 4e). Although the sequences of T13 and T15 had not been deduced, a unique sequence was obtained for the *Hha* I region by combining the sequence information on both strands (Fig. 3, '*Hha* I region').

The five T1 RNase nucleotides remaining (T1c, T3b, T6b, T8 and T12) should be located between the *Hha* I and G3 RNA regions. The sequence of the R·*Hha* cleavage site has been determined (ref. 18 and our unpublished observation). R·*Hha* cleaves DNA at the following sites:



This sequence should be located between the *Hha* I and *Hha* 2 regions. On the basis of this assumption and information on the nucleotides adjacent to G, the order of the five T1 RNase nucleotides can be deduced to be either (a) T3b:T8:T1c:T12:T6b or (b) T3b:T6b:T1c:T12:T8.

The T1 RNase nucleotides produced from (-)RNA·LS are shown in Fig. 4d and Table 1b, in which nucleotides from (-)RNA·LS are denoted by the minus sign. As indicated in Fig. 1, the sequence of (-)RNA·LS should be complementary to the left part of (+)RNA·LS. According to this assumption we arranged the T1 RNase nucleotides of (-)RNA·LS on the sequence of (+)RNA·LS, and 28 nucleotides were successfully ordered on the *Hha* I and G3 regions. Although the sequences of -T14, -T21 and -T22 had not been deduced, analysis of

their pancreatic RNase digests indicates that these fragments can only fit in the indicated regions (Fig. 3). -T23 was assigned to the left end of the sequence, as the yield was less than 1 mol (Table 1b). Only three T1 RNase nucleotides remain (-T3a, -T3b and -T20). These nucleotides were assigned to the region between the *Hha* I and G3 RNA regions. As described above, the five T1 RNase nucleotides of (+)RNA·LS which had been assigned to the corresponding region can be ordered into two alternative sequences ((a) or (b)). The three T1 RNase nucleotides from (-)RNA·LS are only arranged on the sequence of (a), (see Fig. 3). This arrangement was further confirmed by analysis of the portions of (-)RNA·LS hybridised to the *Hha* fragments and of their partial T1 RNase digests. In Fig. 4e, the T1 RNase nucleotides produced from the *Hha* I region of (-)RNA·LS are shown: all the nucleotides assigned to the left of the R·*Hha* cleavage site are seen. The regions covered by other RNA fragments analysed are indicated by the sidelines on the sequence in Fig. 3.

Sequence of RNA polymerase binding site

The initiation complex was formed with *HapC*-*Hae* I in the presence of 0.15 M KCl, and the polymerase-protected fragment was isolated after DNase digestion. The fragment was denatured and RNA synthesis was carried out using conditions described previously¹². AAAC was added as a primer, because RNA synthesis was significantly stimulated by this primer. The synthesised products were fractionated by hybridisation to fd phage DNA, followed by gel electrophoresis. The T1 RNase nucleotides produced from the largest RNA band (Fig. 2d, B1 region) is shown in Fig. 4f. Only six major nucleotides were identified, of which five were -T3a (2 mol), -T3b, -T11 and

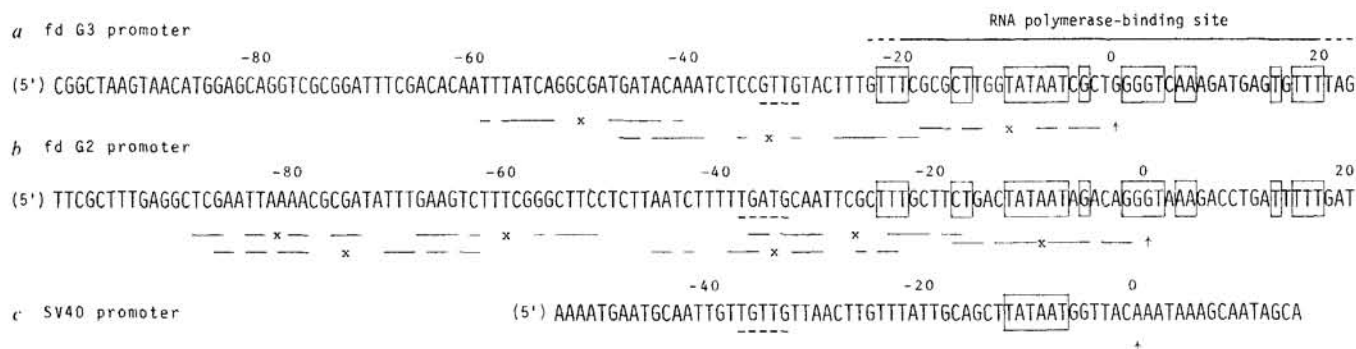


Fig. 5 Comparison of sequences of *a*, fd G3 promoter; *b*, fd G2 promoter, and *c*, a promoter for *E. coli* RNA polymerase on SV40 DNA. The sequences of *b* and *c* were taken from refs 7 and 2. In *a* and *b*, nucleotides common to the polymerase binding sites of both fd promoters are boxed, and the regions with twofold symmetry are indicated by underlining (x, axis of symmetry). In *c*, the homologous sequence in the polymerase binding site is boxed. Arrows indicate the starting points of transcription.

—T20 in the T1 RNase products of (–)RNA·LS. The other (–T22X) contained the primer at 5' end: the result of nearest neighbour analysis indicated that this nucleotide just fits in the region between the sixth and twentieth nucleotides of G3 RNA. It is therefore reasonable to conclude that the RNA analysed had been initiated with the primer in the middle of the –T22 region and terminated within the –T15 region on the sequence of (–)RNA·LS, as indicated in Fig. 3. The size of RNA is about equal to the approximate size of the DNA fragment isolated as the polymerase binding site. The sequence of the polymerase binding site, inferred from the RNA sequence, contains the initiation point for transcription near the centre, in accordance with the observations on other RNA polymerase binding sites^{12–14}.

Sequence features of fd promoter

The sequence in the promoter region for G3 RNA (G3 promoter) inferred from analysis of the low salt transcripts, is shown in Fig. 5*a*. All the information needed for promoter function should be contained in this sequence. The sequence was first compared with the sequence of fd G2 promoter determined previously (Fig. 5*b*)⁷. The longest sequence common to the two similar fd promoters, however, was only TATAAT. It is also seen that when both the promoter sequences are aligned with reference to the TATAAT sequence, a relatively high degree of homology is obtained in the polymerase binding site, as indicated in boxes in Fig. 5*a* and *b*. Any other alignment does not yield such a homology in any region. We previously noted that the G2 promoter contains regions with twofold rotational symmetry, with one located in the non transcribed region of the polymerase binding site (Fig. 5*b*)^{7,12}. The G3 promoter also contains some symmetrical regions (Fig. 5*a*). Comparison of the symmetrical regions, however, does not reveal a common pattern.

In a previous paper⁷, we noted that, among the promoters already sequenced^{1–6}, the G2 promoter sequence bears some resemblance to the sequence of a promoter for *E. coli* RNA polymerase on SV40 DNA (Fig. 5*c*) (TTNTTGNTG at around the –35th position and TATAAT in the polymerase binding site). The G3 promoter also contains some regions homologous to the SV40 promoter (GTTGT and TTGTT at positions –20 and –40 and TATAAT in the polymerase binding site). The sequence common to all three promoters, however, is only the TATAAT sequence. As has been noted by Pribnow¹⁴, the other promoters also contain the sequences homologous to TATAAT in the corresponding region. The result therefore strongly suggests that the TATAAT sequence has an important role for the formation of the initiation complex. It is unlikely, however, that RNA polymerase directly binds to the RNA initiation site by recognising this TATAAT sequence, because the R·Hha cleavage of the G3 promoter five base pairs upstream from the TATAAT sequence destroys the ability to form the initiation complex. It has also been shown that RNA poly-

merase does not initiate RNA synthesis correctly on the isolated polymerase-binding fragment^{14,19}. The exact size of the promoter is not known. In the *lac* and λ promoters, some promoter mutations locate approximately 35 base pairs upstream from the RNA start site^{1,20,21}. In the λ promoters, the R·HindII cleavage site which destroys the promoter function is located 33 base pairs from the RNA start site^{1,5,6}. These observations all suggest that some regions preceding the polymerase binding site are essential for promoter function. It is most likely that RNA polymerase first recognises some specific structure in such a region (recognition site) and then forms an initiation complex at the region containing the TATAAT sequence^{4,14,21}.

The question remains as to the basic structure at the recognition site. One possibility is that some specific sequence is involved in the primary recognition of promoter by RNA polymerase. It has been noted that both the λ and SV40 promoters contain TGTTG about 35 base pairs upstream from the RNA start site²¹. As indicated by broken lines in Fig. 5*a* and *b*, the G2 promoter contains TGATG and the G3 promoter GTTG in the corresponding region, suggesting that this sequence may have some relation to the structure of the recognition site. The *lac*⁴ and tyrosine transfer RNA²⁴ promoters, however, do not contain this sequence. The other possibility for consideration is derived from the present finding that, except for the polymerase binding site, there was little sequence homology between two similar fd promoters. It is known that RNA polymerase has strong affinity towards single-stranded regions of DNA^{22,23}. At low salt concentrations, RNA polymerase binds many sites of DNA²² and initiates RNA synthesis at additional sites, as indicated in this and previous papers⁷. These lines of evidence would lead to a view that RNA polymerase primary interacts with regions of low transition temperature, instead of recognising a specific sequence; and if the TATAAT sequence locates at the neighbouring site, the polymerase forms a stable initiation complex. Such a primary interaction site could be either the region of high AT content or the region with twofold symmetry, as the helical structure of these regions is assumed to be unstable. In support of this possibility, all the promoters sequenced seem to have some AT-rich regions and the fd promoters contain the regions with twofold symmetry in the region preceding the polymerase-binding site, although the location and size of such regions are different with promoters. With either possibility discussed above, more information is still needed to define the basic structure at the recognition site.

Finally, we compared the sequences of two fd promoters with similar properties and found little sequence homology, except for the region in which RNA polymerase forms an initiation complex. As other lines of evidence indicate that the promoter recognition by RNA polymerase takes place at a region preceding the initiation complex site, our finding implies that a unique sequence is not involved as a key element in the promoter recognition.

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Intracellular forms of Epstein–Barr virus DNA in human tumour cells *in vivo*

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Tumour biopsies from Burkitt lymphoma patients, as well as human nasopharyngeal carcinoma cells growing in athymic nude mice, contain Epstein–Barr virus DNA as covalently closed circular DNA. In addition, integrated viral DNA sequences seem to be present.

EBSTEIN–BARR virus (EBV) is of great interest at present as the first virus of possible aetiological significance for certain malignant tumours in man. Two unusual human tumours are uniquely associated with EBV, the African form of Burkitt lymphoma and low-differentiated nasopharyngeal carcinoma. Patients with these tumours always have elevated serum titres against EBV-associated antigens^{1,2}. Moreover, the actual tumour cells are those transformed by the virus, as they contain several copies of EBV DNA and express an EBV-associated nuclear antigen (EBNA) but lack detectable virus particles^{2–5}. Although formal proof that the virus is oncogenic in man will be difficult to obtain, the notion that EBV is a tumour virus is supported by the recent findings that injection of the virus into cotton-top marmosets or owl monkeys induces fatal lymphomas^{6,7}, and that the virus transforms normal human B lymphocytes into immortal lymphoblastoid cell lines with great efficiency⁸. Further, in humans with certain rare forms of inherited immunodeficiency, infectious mononucleosis caused by EBV may proceed into a malignant lymphoma instead of having its usual appearance as a self-limiting lymphoproliferative disease^{9,10}.

Insight into the relationship between EBV and its host

may be gained from studies on the state of the virus genome within the transformed cell. Such investigations have previously been limited to human lymphoid cell lines, in particular Raji cells. This line was derived from a Burkitt lymphoma biopsy over 10 yr ago¹¹. A Raji cell does not produce EBV particles, but it contains 50–60 genome equivalents of EBV DNA¹². Most of this intracellular EBV DNA is present in free form and can be separated from the larger cellular DNA by glycerol gradient centrifugation in both alkaline and neutral solution^{13,14}. This non-integrated EBV DNA has been purified and shown to have a circular conformation^{15,16}, with both covalently closed and nicked circular DNA molecules present after isolation. Thus, the non-integrated intracellular virus DNA differs structurally from the linear duplex DNA present in virus particles, although both these DNA forms are of the same molecular weight within experimental error. In addition, integrated sequences of EBV DNA have been found in Raji cells, but it is not known if they represent complete virus genomes¹⁷. The isolated DNA “molecules” comprised of both viral and cellular DNA usually contain single-strand interruptions, but the actual joints between the viral and cellular DNA are alkali stable¹⁸.

It has not been known if both integrated and circular forms of EBV DNA are also present in tumour cells *in vivo* or if one of these forms was a secondary consequence of *in vitro* cultivation. The studies on the intracellular states of EBV DNA are extended here to tumour biopsies.

Circular EBV DNA in nasopharyngeal carcinoma cells

Biopsies from patients with low differentiated nasopharyngeal carcinoma contain EBV DNA, which is located in the nuclei of the epithelial tumour cells^{19,20}. Such biopsies are usually small and heavily infiltrated with lymphocytes that do not contain EBV DNA, and they are therefore less

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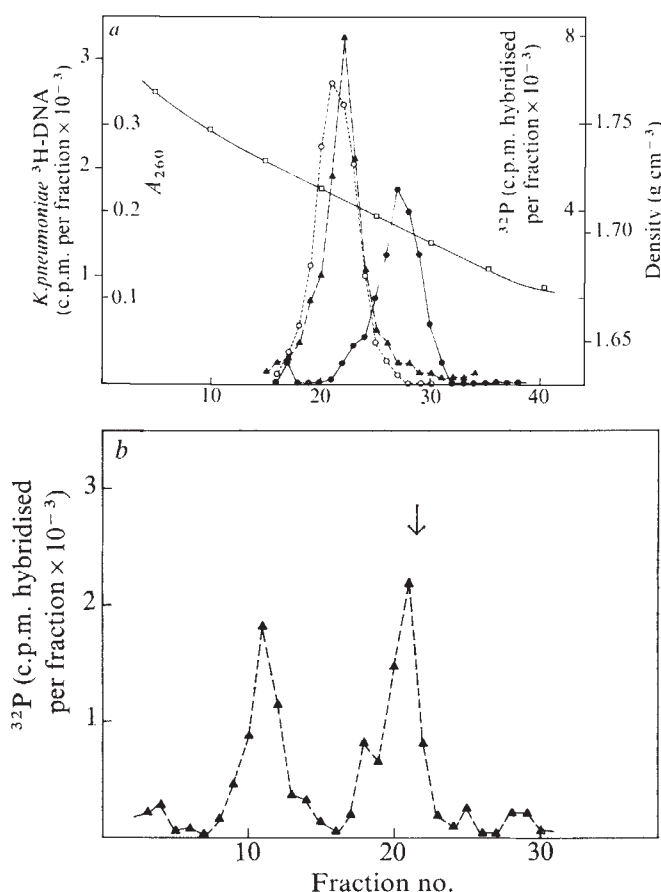
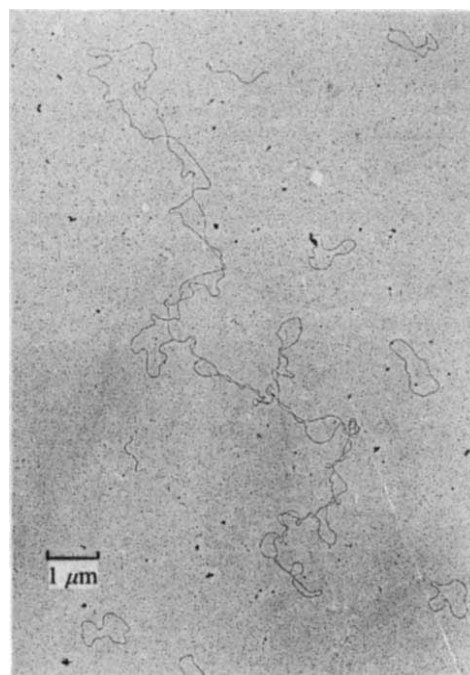


Fig. 1 Separation of EBV DNA and cellular DNA in lysates of nasopharyngeal carcinoma cells. A human tumour (M.M., 1.5 g) growing in a nude mouse was excised, finely minced with a pair of scissors in 6 ml of 0.15 M NaCl–0.01 M Tris–HCl–0.001 M MgCl_2 –0.0005 M CaCl_2 (pH 7.2) at 0°C, and the cells were disintegrated by twenty strokes in a Teflon–glass hand homogeniser. Six ml of 3% Sarkosyl–0.075 M Tris–HCl–0.025 M EDTA (pH 9.0) were then added, and the viscous lysate was brought to 20°C. After 40 min, 3 ml of 0.5% Pronase (pre-incubated for 2 h at 37°C in 0.05 M Tris–HCl (pH 7.5) before use) were added, and the mixture was incubated for 2 h at 37°C. It was then diluted with 100 ml 0.05 M Tris–HCl (pH 8.5), and supplemented with a density marker (*Klebsiella pneumoniae* ^3H -DNA, $\rho = 1.717 \text{ g cm}^{-3}$) and solid CsCl to a final density of 1.714 g cm^{-3} . The material was centrifuged in eight 19-ml aliquots overlaid with paraffin oil in a Spinco 60 Ti rotor at 33,000 r.p.m. for 65 h at 20°C. The gradients were collected through a large hole in the bottom of the tube with a closed system collection device¹⁶ in 0.4-ml fractions. A gradient profile is shown in *a*. In each fraction, the cellular DNA was determined by A_{260} measurements, the DNA density marker by ^3H radioactivity, and EBV DNA sequences by hybridisation of filter-bound DNA with ^{32}P -labelled EBV complementary RNA as described¹⁶. The density of every fifth fraction was determined by refractometry. ●, A_{260} ; ○, *K. pneumoniae* ^3H -DNA; ▲, EBV DNA; □, density. High density DNA from a second nasopharyngeal carcinoma (J.G.) was isolated from two gradients of the type shown in frame *a* (fractions 20–23), dialysed against 0.1 M NaCl–0.01 M Tris–HCl (pH 8.0)–0.001 M EDTA, concentrated twofold by packing the dialysis bag in dry polyethylene glycol 6000 for 50 min, mixed with a size marker, phage T4 ^{14}C -DNA (0.6 μg , 9,000 c.p.m.), and applied to a 37-ml glycerol gradient (10–30%) containing 1 M NaCl–0.02 M Tris–HCl (pH 8.0)–0.001 M EDTA. The DNA was centrifuged in a Spinco SW27 rotor at 25,000 r.p.m. for 220 min at 21°C, and the centrifuge profile is shown in *b*. 1-ml fractions were collected, the T4 DNA marker (arrow) was localised by radioactivity measurements, and the EBV DNA sequences (▲) by hybridisation with EBV ^{32}P -cRNA. The direction of sedimentation is from right to left.

tumours growing in nude mice to investigate the intracellular state of EBV DNA in nasopharyngeal carcinoma cells.

The M. M. and J. G. carcinomas contain about 80 and 150 EBV genome equivalents per tumour cell, respectively²⁰. Tumour-carrying nude mice were transported from the Houston laboratory to Stockholm. The animals were killed, and the tumours were immediately excised and used for the preparation of high molecular weight DNA. The DNA was then fractionated by CsCl density gradient centrifugation and collected avoiding shearing forces. The EBV DNA sequences were localised by hybridisation of the individual gradient fractions with ^{32}P -labelled EBV complementary RNA. The experimental techniques are the same as those previously used with Raji cells^{15–18}. A typical gradient profile is shown in Fig. 1. EBV DNA has a higher guanine–cytosine content than cellular DNA, and most of the virus DNA sequences are separated from the bulk of the cellular DNA and form a peak at a density of 1.716 g cm^{-3} . The EBV DNA peaks from two CsCl gradients were pooled and further fractionated by neutral glycerol gradient centrifugation together with phage T4 ^{14}C -DNA as an internal size marker²¹. Linear EBV DNA from virus particles sediments at 58–59S in these conditions^{15,16}. In contrast, the intracellular EBV DNA sequences from the J. G. and M. M. tumours were found as two distinct peaks at about 101S and 64S (Fig. 1). The two forms of intracellular EBV DNA from the nasopharyngeal carcinoma cells sediment at 1.7 and 1.1 times the rate of linear EBV DNA from virus particles, indicating that they correspond to covalently closed circular virus DNA molecules and open circular virus DNA molecules, respectively²². The presence of large covalently closed circular DNA molecules was directly confirmed by electron microscopy

Fig. 2 Electron micrograph of a large, covalently closed circular DNA molecule from a nasopharyngeal carcinoma tumour (M.M.). The EBV DNA was partly purified by CsCl density gradient centrifugation and glycerol gradient centrifugation. The DNA sedimenting at 95–110S in the latter type of gradient was pooled and dialysed against 0.05 M NaCl–0.01 M Tris–HCl (pH 8.1)–0.005 M EDTA and spread by a modification of the microdiffusion technique avoiding the use of formaldehyde^{16,25}. The small DNA circles in the figure are phage PM2 DNA molecules. In order to partially untwist the supercoiled molecules, $4 \mu\text{g ml}^{-1}$ ethidium bromide was included in the DNA–cytochrome *c* solution. For length measurements, however, only preparations without ethidium bromide were used.



suitable for biochemical work. The tumour cells carrying EBV, however, continue to grow after implantation to congenitally athymic (nude) mice, whereas the infiltrating lymphoid cells disappear²⁰. We have used such human

(Fig. 2). Such large DNA circles have previously been found in corresponding fractions from Raji cells, but are not detected in EBV-negative human cell lines¹⁶. The covalently closed circular DNA molecules from nasopharyngeal carcinoma cells (J. G.) were converted to an open circular form by X-ray treatment, mixed with open circular DNA from phage PM2 (a gift from Dr R. Streeck) as an internal size marker, and used for contour length measurements on electron micrographs¹⁶. These EBV DNA circles were found to be 16.2–16.3 times larger than PM2 DNA molecules, corresponding to an approximate molecular weight of 104×10^6 . In previous measurements on circular EBV DNA from the Burkitt lymphoma derived cell line Raji, the molecules were found to be 16.6 times larger,

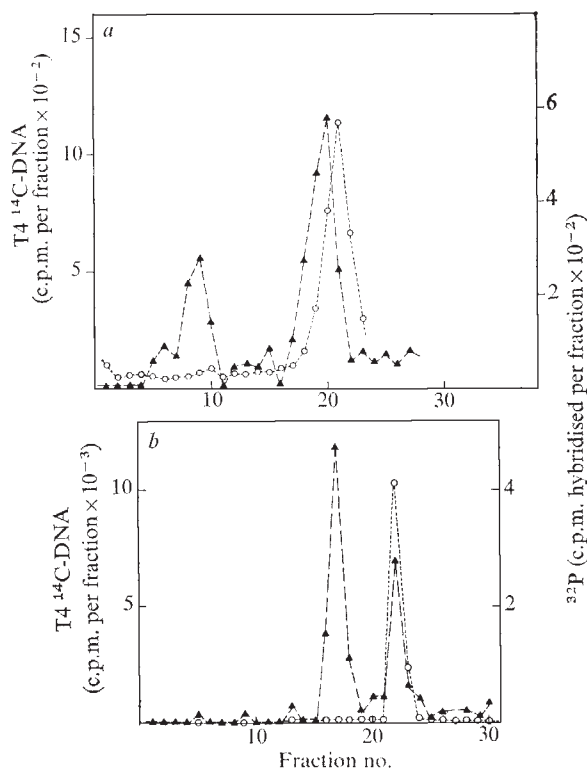


Fig. 3 Fractionation of high density DNA from a Burkitt lymphoma biopsy. The A.O. biopsy (5 g) was minced with scissors, and the material was suspended in ten volumes tissue culture medium by shaking. After 10 min at 21 °C, the material that had settled to the bottom, which consisted largely of stroma, was discarded, and suspended cells were collected by low speed centrifugation and washed in phosphate-buffered saline. The cells (10^7 cells ml^{-1}) were lysed by addition of Sarkosyl-EDTA and Pronase, and the DNA was fractionated by CsCl gradient centrifugation as in Fig. 1a. The high density DNA was collected and cosedimented with phage T4 DNA on a glycerol gradient as in Fig. 1b. The results from the latter gradient are shown in frame a. ▲, EBV DNA, as determined by hybridisation with EBV cRNA; ○, T4 DNA. The fractions 25–31 contained the density marker DNA from the CsCl purification step. The 105S DNA from one neutral glycerol gradient was recovered, mixed with linear ¹⁴C-DNA (from phage T4), and dialysed against 1 M NaCl–0.02 M Tris-HCl (pH 8.0)–0.001 M EDTA for 20 h, followed by 0.05 M NaCl–0.02 M Tris-HCl (pH 8.0)–0.001 M EDTA for 3 h. The material was concentrated two- to threefold by packing the dialysis bag in dry polyethylene glycol 6000 for 1 h, followed by an additional 3-h dialysis against the last buffer. The DNA sample (2.2 ml) was then supplemented with 0.2 ml 0.6% propidium diiodide in 0.01 M Tris-HCl (pH 8.0) and 2.25 g solid CsCl. Centrifugation was carried out in a 5-ml tube overlaid with paraffin oil in a Spinco SW 50.1 rotor at 40,000 r.p.m. for 48 h at 21 °C. Fractions (0.1 ml) were collected from the bottom of the tube, and the fractions were freed from the dye by four extractions with two volumes of 90% isopropanol. Data are shown in frame b. ○, T4 DNA; ▲, EBV DNA, as determined by hybridisation with EBV cRNA.

than PM2 DNA¹⁸, and the covalently closed and open circular forms of the virus DNA has sedimentation coefficients of 103–105S and 65S, respectively. Thus, both the contour length measurements and the sedimentation velocity data indicate that the circular EBV DNA in nasopharyngeal carcinoma cells may be slightly smaller than that in Raji cells, suggesting the presence of a different EBV subgroup or a small deletion of the virus DNA in the epithelial cells.

Circular EBV DNA in Burkitt lymphoma biopsies

Burkitt lymphoma patients were treated by tumour excision in Nairobi. Tumour material was transported on ice to Stockholm, and was available for experimentation about 24 h after excision. Burkitt lymphoma biopsies are more homogeneous than nasopharyngeal carcinoma biopsies, and occasionally large specimens with a high proportion of living tumour cells (>80%) became available. Two such tumours were investigated, an ovary tumour from a 9-yr-old girl (A.O.) and a tumour excised from the submandibular region of a 4-yr-old boy (W.J.).

High molecular weight DNA was extracted from the Burkitt lymphoma cells and fractionated on CsCl gradients and neutral glycerol gradients. The results were quite similar to those obtained with the nasopharyngeal carcinoma cells. With material from one of the biopsies (A.O.), two distinct peaks of EBV DNA at 105S and 65S were observed by hybridisation of the glycerol gradient fractions with EBV complementary RNA (cRNA) (Fig. 3), representing covalently closed circular EBV DNA and open circular EBV DNA. In the other biopsy, a single peak of 65S EBV DNA was found, corresponding to open circular DNA. The apparent absence of covalently closed circular EBV DNA in the latter case may have been due to the introduction of single-strand breaks into the viral DNA after tumour excision, either during the transport to Stockholm or during the preparation of the lysate from the tumour.

The 105S and 65S EBV DNA fractions from the A.O. biopsy were further characterised by equilibrium density gradient centrifugation in propidium diiodide–CsCl gradients. Covalently closed circular duplex DNA has a higher density in such gradients than open circular or linear DNA²³. Most of the EBV DNA (70%) from the 105S region banded as a distinct peak at a position typical of covalently closed circular DNA (Fig. 3), as estimated from parallel control experiments with polyoma virus DNA. The rest of the EBV DNA (30%) was found at the position of open circular and linear DNA, and was presumably due to DNA damaged during the preparation procedures. All of the 65S EBV DNA banded as open circular or linear EBV DNA.

Integrated EBV DNA

Part of the EBV DNA sequences present in Raji cells band at an anomalously light density in CsCl gradients. The densities of these viral DNA sequences remain unchanged after treatment with Pronase, sodium dodecyl sulphate, and phenol, but they appear at a heavier density after reduction of the size of the DNA preparations by shearing forces. These data indicate that integrated EBV DNA sequences are present in Raji cells^{17,18}. A similar experimental approach has now been used with the tumour biopsy material studied here. In the nasopharyngeal carcinoma DNA shown in Fig. 1a, a small part of the total EBV DNA sequences is present under the peak of cellular DNA. To remove EBV DNA tailing into the low density region, this DNA (fractions 26–29 in Fig. 1a) was rebanded on a second CsCl gradient. The DNA banding at a density of 1.698–

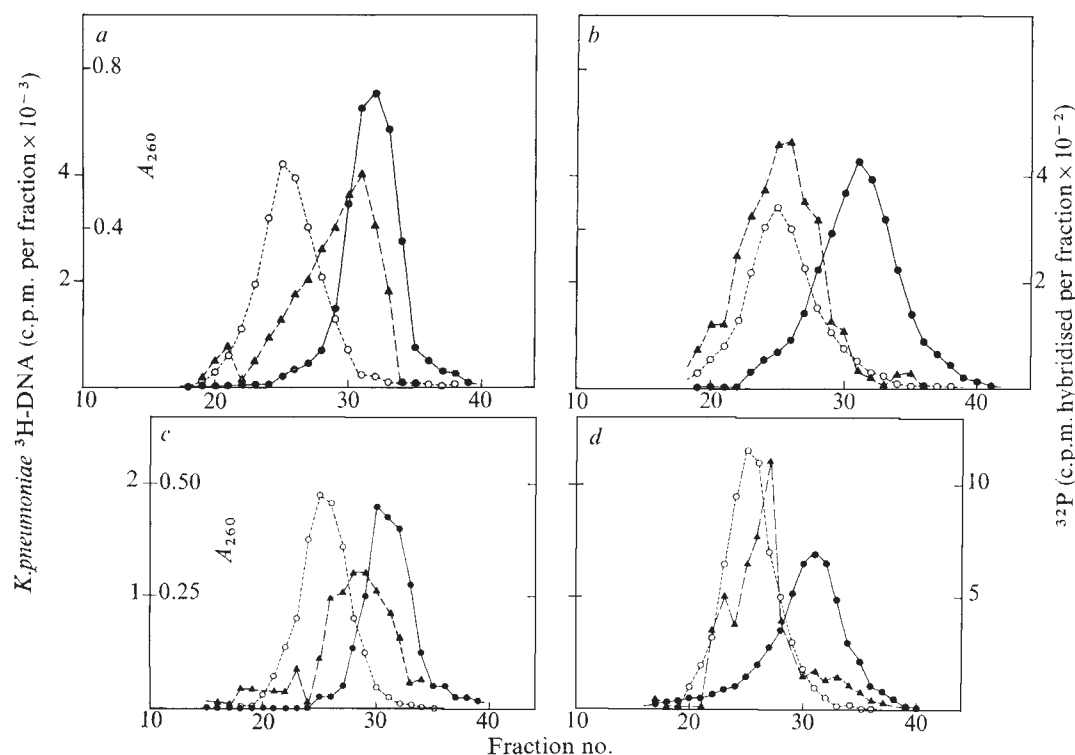


Fig. 4 Third sequential fractionation by CsCl gradient centrifugation of cellular density DNA from tumour cells. For studies on nasopharyngeal carcinoma cells, fractions 26–29 in Fig. 1a were rebanded in CsCl in the same experimental conditions, and the main peak of A_{260} absorbing material from the second gradient was pooled, mixed with a density marker (*K. pneumoniae* ^3H -DNA), and divided in two parts. One part was sheared by three rapid passages through a No. 25 hypodermic syringe needle. The two DNA solutions were individually rebanded in CsCl in the same experimental conditions. *a*, Unsheared DNA; *b*, Sheared DNA. ●, A_{260} ; ○, *K. pneumoniae* ^3H -DNA; ▲, EBV DNA sequences determined by hybridisation with EBV ^{32}P -cRNA. The same experimental design was used with Burkitt lymphoma biopsy cells, and the data obtained with the A.O. biopsy are shown in frames *c* and *d*. After purification of cellular density DNA by two consecutive CsCl gradient centrifugation steps, the material was divided in two parts, and the size of the DNA of one part was reduced twentyfold by shearing before rebanding and hybridisation. *c*, Unsheared DNA, and *d*, sheared DNA. Symbols as in *a* and *b*.

1.708 g cm^{-3} in this gradient was supplemented with a density marker (*Klebsiella pneumoniae* ^3H -DNA) and divided in two equal parts. One part was again centrifuged to equilibrium in neutral CsCl. Most of the remaining EBV DNA sequences in this DNA fraction now banded at a peak density of about 1.704 g cm^{-3} , but some EBV DNA sequences of heavier densities were still present (Fig. 4a). The other part of the material was reduced in size by shear treatment, and then banded in CsCl in the same conditions. In this case, the EBV DNA sequences were released to heavier densities. (Fig. 4b). This is the expected result if large DNA molecules containing both EBV DNA sequences and cellular DNA sequences had been fragmented by the shear treatment.

The DNA preparations from the two Burkitt lymphoma biopsies were also studied by the same techniques, and the results were essentially identical to those obtained with the nasopharyngeal carcinoma material. With both biopsies, a low density fraction of EBV DNA sequences could be isolated by three consecutive bandings in CsCl, and these viral DNA sequences were shifted to heavier densities by reduction of the size of the DNA. The data obtained with one of the biopsies (A.O.) are shown in Fig. 4. It is clear that this type of low density EBV DNA, which was first detected in lymphoid cell lines and presumably consists of integrated sequences of virus DNA, is present also in tumour biopsies of Burkitt lymphoma and nasopharyngeal carcinoma origin. Thus, the integrated viral sequences can not be ascribed to chromosome translocation events or other artefacts of long term *in vitro* culture.

Episomal state of latent EBV

In bacteria, sex factors and certain prophages can be carried both in an integrated state and as stable extra-

chromosomal elements in the form of circular DNA molecules. The discovery of circular EBV DNA molecules in virus-transformed human cells, free from virus particles, strongly indicates that episomes also occur among mammalian cells. Little information is available at present on the carrier states of other large animal viruses, and it seems possible that all herpesviruses may be carried as episomes during latency. The relationship between the non-integrated EBV DNA circles and the integrated EBV DNA sequences found in transformed cells remains a subject for future study.

In addition to the investigations on tumour cells, we have recently shown (to be published) that circular EBV DNA molecules are also found in EBV DNA-carrying lymphoblastoid cell lines derived from normal individuals and from infectious mononucleosis patients. A lymphoblastoid cell line derived from a normal individual also contained EBV DNA sequences with the properties of integrated DNA. No clear difference has so far been observed between the carrier state of EBV in cell lines from normal individuals and in tumour cells. A simple model in which either integrated EBV DNA sequences or circular EBV DNA molecules would only be found in tumour cells is not in agreement with the available data. It should be noted, however, that EBV-carrying lymphoblastoid cell lines derived from human blood of healthy donors have malignant potential, as they cause tumours on implantation in nude mice²⁴.

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letters to nature

New H₂O celestial sources associated with H II regions in the Southern Hemisphere

WE report here the first results of a nearly complete, high-sensitivity, water vapour survey of galactic H II regions situated in the Southern Hemisphere.

A typical upper limit of about 40 Jy (1 Jy = 10^{-26} W m⁻² Hz⁻¹) was attained at the $6_{16} \rightarrow 5_{23}$ rotational transition line of the water vapour molecule (that is, a frequency of 22,235.08 MHz).

A new ruby travelling wave K-band maser receiver¹ was used at the 13.7-m Itapetinga radio telescope², Atibaia, São Paulo, Brazil, between November 4 and 8, 1975. The Dicke-switched system temperature was typically 350 K, including radome contribution (less than 10 K)³ and sky contribution (less than 100 K during this season)³. The back-end section of the receiver consisted of a 43-channel spectrograph with 100-kHz filters. Observations were made using a combined beamswitching and 'on-on' technique.

The 13.7-m Itapetinga radio telescope has an absolute pointing accuracy better than 15'' arc, which is negligible in comparison with its 4.5' arc beamwidth at that frequency. The aperture efficiency was redetermined using Virgo A (assuming 21.4 Jy)⁴ and Venus (brightness temperature of 420 K)⁵ as calibrators, and was found to be 34.5 Jy K⁻¹ (that is, 54% efficiency).

Observations were carried out during the worst season for millimetric work at Itapetinga. Frequent measurements of atmospheric absorption were necessary. Optical depths were determined using the sky emission method³, and ranged from 2.61 to 4.34 dB. All measurements were corrected for atmospheric and radome absorption, the latter being 23% at 33.2 GHz.

The most complete published surveys of new H₂O celestial sources in the Southern Hemisphere are those by Johnston *et al.*⁶, and by Caswell *et al.*⁷. They reported sources having fluxes larger than or equal to about 200 Jy.

We examined all 47 of the individual H II regions with 5 GHz continuum peak brightness levels of 5 K or more, as reported by Goss and Shaver⁸, and all 24 regions for which OH main-line emission has been found^{9,16}.

Observations were made at reported positions (Table 1), and no attempt was made to find improved H₂O positions.

Thirteen new H₂O celestial masers were found (Table 1). The spectrograph was centred at the velocities of associated OH lines, when known, otherwise at H109 α recombination lines¹². The search width for each observation was in general ± 29 km s⁻¹.

Of the sources associated with OH emission, all except H₂O 0.55–0.85 had previously been examined^{6,7} for H₂O emission. It is generally accepted that sources having OH main-line emission are also likely to show H₂O emission¹³. In Fig. 1a we show the spectrum for the strongest source discovered in this class. Another interesting H₂O source, with three line components, is H₂O 0.55–0.85. This has been identified with RCW142 (Fig. 1b), a dense molecular cloud near the galactic centre¹¹.

The seven new H₂O sources associated with H II regions that do not exhibit OH emission, suggest the existence of a new class of celestial water vapour masers. Figure 1c shows the spectrum of H₂O 316.8–0.1, the most intense source found in that class during our survey. Another interesting member of that class of sources is H₂O 291.7–0.7 (Fig. 1d). At least one strong emission line can be observed at –32.8 km s⁻¹, with an intensity of 159 Jy superimposed on a 1.8-K continuum level. That source, and H₂O 267.9–1.1 (identified with RCW38), are known to be associated with compact protostellar

Fig. 1 a, H₂O 345.5+0.3, the strongest source found in association with OH main-line emission (spectrum is average of three 6-min runs); b, spectrum of H₂O 0.55–0.85 (RCW142), (integration time, 6 min); c, H₂O 216.8–0.1, the strongest source not associated with OH main-line emission (spectrum is average of two 6-min runs); d, H₂O 291.3–0.7, associated with the extended H II region RCW57, which contributes the 1.8-K continuum level (spectrum is the average of four 6-min runs).

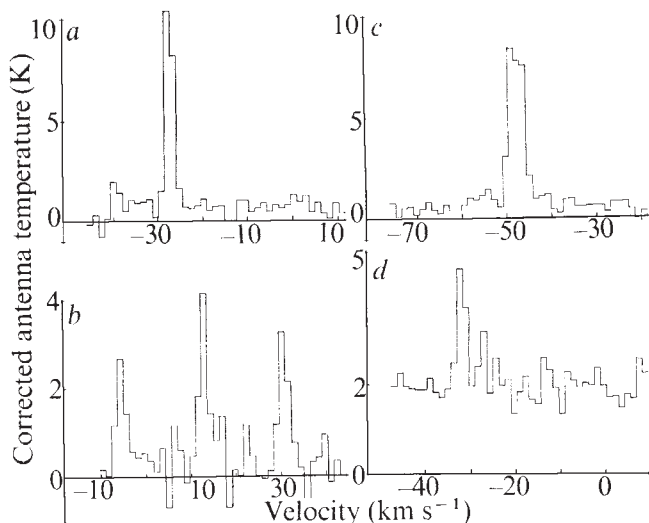


Table 1 New H₂O celestial masers in Southern Hemisphere H II regions

Galactic coordinates	H ₂ O line peak intensity in Jy (November 1975)	Radial velocity at peak intensity (km s ⁻¹ in relation to LSR)	Reference for p positions	Comments
Sources associated with OH main-line emission				
0.55-0.85	128	+11.6	11	RCW142. One 6-min run, centred at +13 km s ⁻¹ .
330.0-0.4	45	-67.2	9	Three 6-min runs, centred at -60 km s ⁻¹ .
333.2-0.1	59	-84.6	10	Two 6-min runs, centred at -90 km s ⁻¹ .
338.9+0.6	127	-76.3	9	Two 6-min runs, centred at -60 km s ⁻¹ and -75 km s ⁻¹ .
345.5+0.3	359	-28.1	9	Three 6 min runs, centred at -20 km s ⁻¹ . See Fig. 1.
347.6+0.2	49	-83.8	9	Two 6-min runs, centred at -100 km s ⁻¹ . Another possible weaker line at -94.6 km s ⁻¹ .
Sources at regions that do not exhibit OH main-line emission				
267.9-1.1	171	-6.1	8	RCW38. One 6-min run, centred at +2 km s ⁻¹ .
291.3-0.7	159	-32.8	8	Four 6-min runs, centred at -22 km s ⁻¹ . See Fig. 1d.
305.3+0.2	79	-42.7	8	Three 6-min runs, centred at -40 km s ⁻¹ . Another possible 53 Jy, line at -40 km s ⁻¹ .
316.8-0.1	290	-48.6	8	Two 6-min runs, centred at -50 km s ⁻¹ . See Fig. 1c.
326.7-0.6	70	-41.3	8	Three 6-min runs, centred at -20 km s ⁻¹ ; two 6-min runs, centred at +5.2 km s ⁻¹ . Other lines at -7.0 km s ⁻¹ (60 Jy), -13 km s ⁻¹ (66 Jy), and -62 km s ⁻¹ (61 Jy).
332.7-0.6	58	-47.3	8	Two 6-min runs, centred at -50 km s ⁻¹ .
348.7-1.0	155	-12.7	8	Two 6-min runs, centred at -10 km s ⁻¹ . Another possible 65 Jy line at -31.6 km s ⁻¹ .

infrared objects¹⁴. Another source found, tentatively designated as H₂O 333.0-0.4, and showing a 64-Jy line at -51.3 km s⁻¹, is possibly a confusion with a known nearby water vapour source, H₂O 333.2-0.5, which has OH main-line emission⁷.

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Observations of Cyg X-1 from Aryabhata

ARYABHATA, India's first artificial satellite, which was launched into a near circular orbit of 600 km altitude at an inclination of 51° on April 19, 1975, carried instruments to investigate celestial X rays in the energy range 2.5-155 keV. We report here observations of Cyg X-1 made in the range 2.5-18.75 keV, during the second orbit of the satellite, centred around 108 d 9 h 17 min 40 s UT, just before the source made a major increase in its intensity. We found a hardening in the spectrum.

We investigated this energy range with a proportional counter telescope filled with a mixture of Ar, CO₂ and He in the pressure ratio of 90:9.5:0.5 to a total pressure of 1 atm. The counter, which had a 2.5 0.0025-inch Be entrance window and an effective area of 15.4 cm², was fitted with cylindrical aluminium collimators for restricting the field of view to 12.5° FWHM. This telescope was mounted with its viewing direction along the spin axis of the satellite so that it could be oriented stably and operated in the pointed mode. The sighting on Cyg X-1 was made possible before the spacecraft was spin stabilised and during the time when it was performing an extremely slow spin around an axis different from the actual spin axis.

The counting rates were monitored in four energy channels: 2.5-5.4 keV, 5.4-9.8 keV, 9.8-13.8 keV and 13.8-18.75 keV, thus enabling the spectral determination of the source. Background rates were evaluated from the study of the counting rate profile as a function of time as well as their known dependence on the geographical latitude of the satellite. The attitude of the X-ray detector in space was computed using the outputs from magnetometers and Sun sensors mounted on the satellite. The excess rates corresponding to Cyg X-1 were corrected for the offset angle of the telescopic axis with respect to the

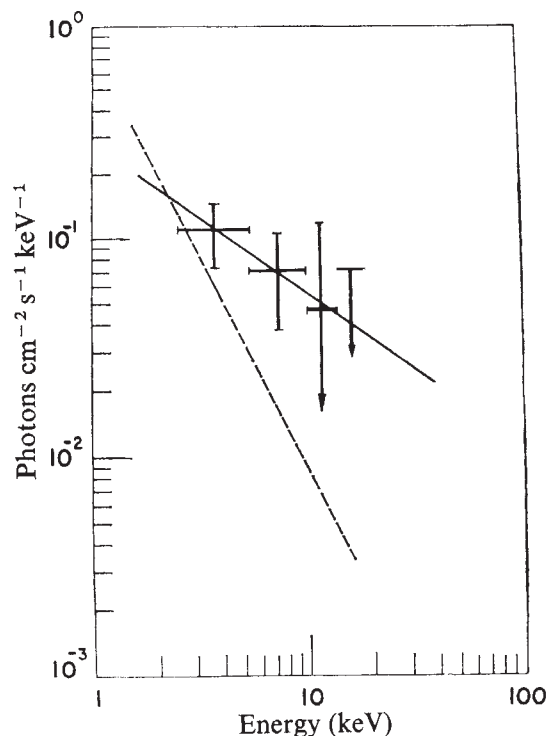


Fig. 1 The spectrum of Cyg X-1, as observed on 108 d 9 h 17 min 40 s UT (April 19, 1975), before the intensity increase. The dotted line gives the spectrum observed by Ariel V on May 13, 1975.

source direction and for the efficiency of the counter as a function of energy. The photon spectral distribution so derived is shown in Fig. 1. The spectrum shows a power law dependence with an index of $\sim 0.7 \pm 0.2$ in the energy interval of 2.5–13.8 keV, the estimated intensity being 0.84 ± 0.3 photon $\text{cm}^{-2} \text{s}^{-1}$ at the time of observation.

Cyg X-1 underwent a major upward transition in its intensity during late April 1975, as revealed by the detectors on the Ariel and ANS satellite¹⁻³. This intensity transition seems to be the reverse of the one observed by Uhuru⁴ in April 1971. A distinct feature of these intensity transitions is the accompanying spectral change⁵, with the spectral exponent changing from the value of 1.6 observed during the normal state to 2.2 in the 'high' state. The all-sky camera on Ariel V (ref. 2) seems to place the occurrence of the intensity transition around the latter half of April 22, 1975 based on long term intensity monitoring for this source in the 3–6 keV energy range from November 1974 to May 1975. The results presented here complement the results of the all-sky camera by providing definitive spectral information, for the first time, just before the transition. Thus, together with Ariel observations, it is now possible to examine the physical state of this X-ray source just before and just after this low-high transition. Whereas the intensity from Cyg X-1 registered by our detector in the 3–6 keV range ($\sim 0.3 \pm 0.1$ photon $\text{cm}^{-2} \text{s}^{-1}$) is comparable with that of all-sky camera ($\sim 0.4 \pm 0.1$ photon $\text{cm}^{-2} \text{s}^{-1}$), the spectrum observed by us is harder than the typical one for this source, even in its normal state. For purposes of comparison, the spectral nature of the source after the upward intensity transition as observed by Ariel V (ref. 3) is also shown in Fig. 1. It is, however, to be noted that the spectral hardening of this type with time scales of the order of minutes has been reported¹ previously also for Cyg X-1 in its normal state. Whether such spectral hardening forms a prelude to the transition of the source or is a property of its short term fluctuations, not necessarily related to the transition, needs further investigation.

Cyg X-1 is an interesting and complex X-ray source, probably in a binary system, where X-ray emission takes place during the mass transfer from the OB supergiant to a compact companion.

This source is known to exhibit intensity variations with time scales of the order of milliseconds, minutes and even days^{5,6}. Flare-like enhancements⁷, sudden intensity transitions⁴ and intensity dips around superior conjunction of the spectroscopic binary have also been observed.

Attempts have been made to understand the intensity transition in Cyg X-1 in terms of the variable mass accretion from the OB supergiant by the compact companion⁸ and the possible instabilities in the accretion disk⁹. Dips in intensity observed in association with the superior conjunction of the binary¹⁰, resulting in spectral hardening, can arise from discrete absorption features such as gas streams of localised character¹¹. Such features can also explain short term hardening of the spectrum of the type reported here. A single self-consistent explanation that can account for all the emission characteristics of Cyg X-1 has, however, yet to emerge.

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Intrusions and double-diffusive convection

A STRATIFIED fluid layer in which two components contribute to the vertical density distribution need not be stable even though the net density decreases upwards. If one of the components is unstably distributed, then molecular diffusion can release its potential energy—a phenomenon known as double-diffusion convection¹. Considerable progress has been made over the past 10 years in the study of double-diffusive convection in situations where the properties vary in one direction (vertically) only. In the ocean, horizontal advection is nearly always important and its neglect in the previous laboratory experiments makes it difficult to apply the results in a wider context. A first step in the study of two-dimensional effects in double-diffusive convection was made by Turner and Chen², and here I follow up one of their suggestions in detail in the hope that it may help to understand the layering which occurs when an outflow intrudes into an ambient fluid with the same density but different component concentrations³.

Convection (when driven by double-diffusive processes) occurs in well mixed layers separated by comparatively thin regions of high density gradients, called interfaces. The interface can take either of two forms depending on whether the potential energy, which drives the motion, is provided by the component of higher or lower diffusivity. In the former case a 'diffusive' interface is formed; in the latter case a 'finger' interface¹. Layering that can be asso-

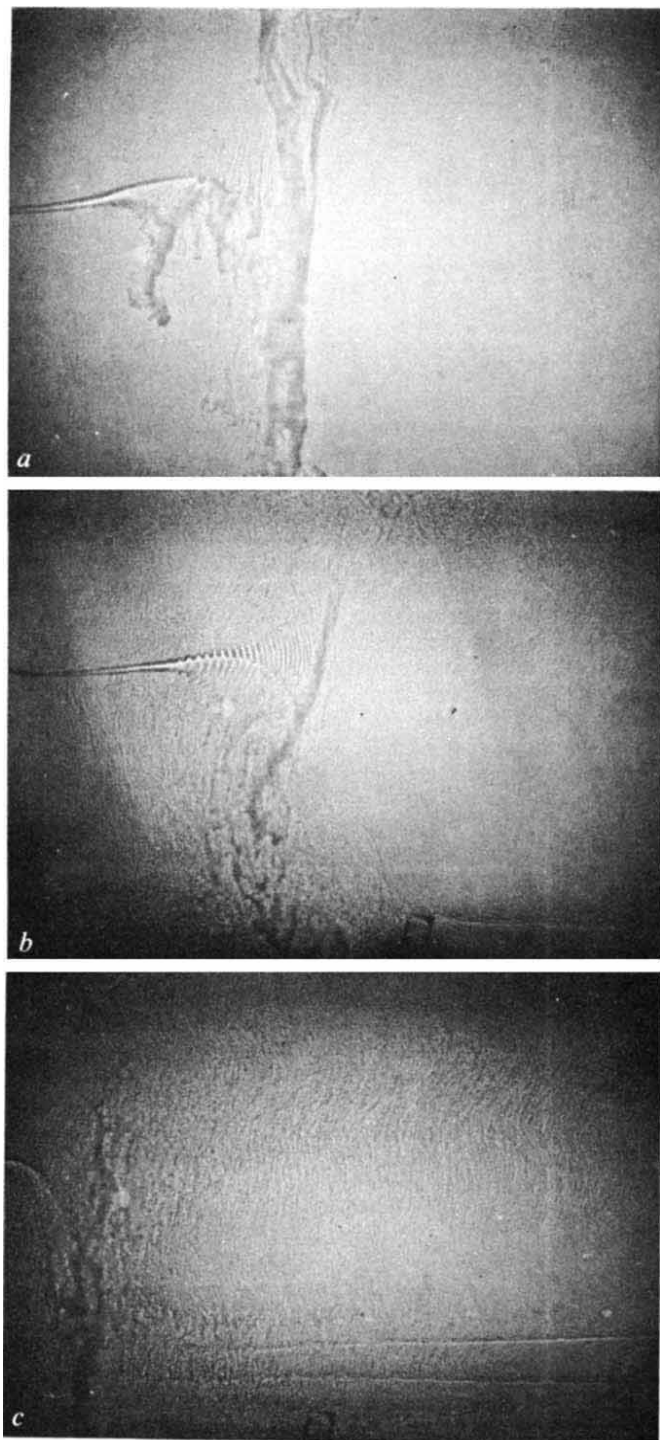


Fig. 1 The behaviour of a sugar intrusion (density, 1.180 g cm^{-3}) into a salt solution (same density): *a*, after 30 s; *b*, after 1 h; *c*, after 2 h.

ciated with both types of double-diffusive processes has now been observed in the ocean.

The experiments were performed in a transparent tank 98 cm long, 29 cm deep and 15 cm wide. The flow was observed using the shadowgraph technique. A small source (internal diameter 1 mm) was located at one end of the tank at mid-depth and the fluid was released in an approximately horizontal direction. Some preliminary experiments were performed in which a salt solution was released into an homogeneous salt solution of the same density (1.180 g cm^{-3}). The flow rate was adjusted so that the motion remained laminar. The released fluid was found to propagate smoothly across the tank with very little mixing. The same flow rate was used in all subsequent experiments.

Two types of experiment were performed, and because of their similarity only one is described in detail here. In the first type of experiment the tank was filled with an initially homogeneous salt solution with a density of 1.180 g cm^{-3} , and a sugar solution was released. The density of the sugar solution was adjusted until it too was 1.180 g cm^{-3} , and then minute adjustments were made so that when released into the salt solution it initially moved horizontally. The sugar solution was left flowing for periods of up to 3 h.

Figure 1*a* shows the behaviour of the plume after 30 s. The downward motion on the left hand side of the main vertical motions was caused by unsteadiness in turning on the intrusion. The plume becomes denser (because the diffusivity of salt is greater than that of sugar) and sinks. The immediate surroundings become less dense as they lose more salt than they gain sugar, so they rise. Thus vertical motions are generated in both upward and downward directions, thus stratifying the tank. Figure 1*b* was taken after 1 h. A diffusive interface can be seen near the bottom of the tank, and fingers can be seen growing from the top of the tank. The downward plume comprises sugar with a little salt, and as it is denser than the ambient fluid it spreads out along the base of the tank. Thus, near the base the salt gradient is destabilising, and as it is the component with greater diffusivity, a diffusive interface is formed.

The upward plume carries a reduced amount of salt (in comparison with the ambient concentration) and a little sugar. This plume spreads out on the surface. The sugar has a destabilising effect, and fingers are formed near the surface. The regular vertical striations which can be seen on the upper edge of the plume near the bending point were stationary and had the appearance of crests in a standing wave pattern. They are also evident in Figs 1*a* and 2*a*. Figure 1*c* was taken after 2 h and shows the final stage in the experiment. There is now extensive fingering in the upper half of the tank and several diffusive interfaces in the lower half. These double-diffusive processes will further stratify the fluid.

Thus, strong vertical motions are generated, carrying excess sugar to the surface ('excess' in this context means a more concentrated solution than that immediately below the surface), and reduced salt to the base of the tank. These concentration profiles provide available sources of potential energy which drive the double-diffusive processes. The vertical motions stratify the fluid and the double-diffusive processes further stratify it.

In the second type of experiment a salt solution, with a density of 1.180 g cm^{-3} , was released into a sugar solution of the same density. Again, strong vertical motions were generated; however, this time the released solution rose carrying an excess of salt to the surface, and the downward plume carried a reduced sugar concentration to the base. These concentration profiles produced diffusive interfaces at the surface of the tank, and fingers at its base. These features are illustrated in Fig. 2*a* which was taken after 1 min, and in Fig. 2*b* which was taken 2 min after the experiment ended (about 2 h after Fig. 2*a*).

The Mediterranean outflow consists of hot salty water which intrudes at its own density level into the cooler, less salty Atlantic Ocean, forming a salinity maximum at 1,200 m. Layering consistent with the finger process has been found beneath the Mediterranean outflow³ and layering stratified in the opposite sense has been found above it. Williams⁴, using a sophisticated optical system has photographed salt fingers below the Mediterranean outflow, thus increasing confidence in the double-diffusive explanation of the layering. In the Mediterranean outflow the sites for the initiation of double-diffusive convection occur at the upper and lower interfaces of the intruding wedge of hot salty water. At the upper boundary, heat is the destabilising

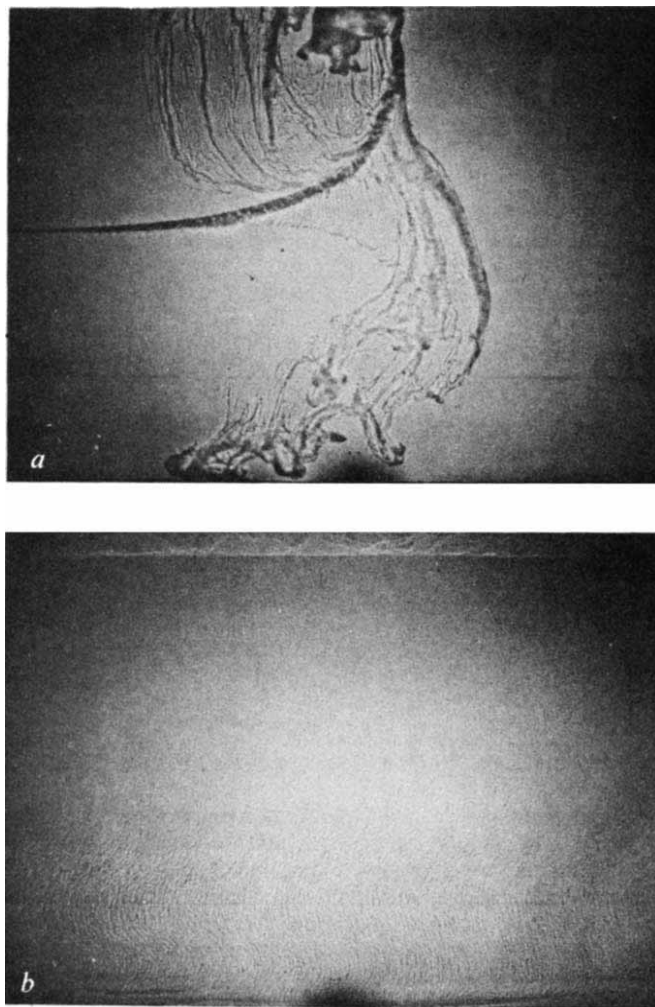


Fig. 2 The behaviour of a salt intrusion (density, 1.180 g cm^{-3}) into a sugar solution (same density): *a*, after 1 min; *b*, 2 min after end of experiment.

component giving diffusive interfaces, and at the lower boundary, salt is the destabilising component giving fingers. In the laboratory, the vertical plumes produce the destabilising gradients at the upper and lower boundaries of the tank. The oceanic situation could be modelled more accurately by using a two-component solution (initially homogeneous, then with both gradients stabilising, and finally with either gradient destabilising) and a two-component intrusion with different concentrations but the same density as at the release depth.

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New Zealand temperatures since 1300 AD

THE period 1900–1935 was the coldest in New Zealand in recorded history. A climatic amelioration was experienced throughout the whole country between 1935 and 1970, with temperatures climbing by 1°C over those years¹. It is interesting to examine recent glacial behaviour in the light of these facts.

New Zealand west coast glaciers, such as the Fox and Franz Josef glaciers, are fast moving because of their steep gradients; they are also nourished by high precipitation rates. As a result, they respond rapidly to climatic fluctuations; however, glaciers are complex systems, so conclusions regarding climatic events must be treated cautiously. The west coast glaciers examined here descend to low altitudes (300 m) and flow rapidly, so the position of their terminal faces can be used as climatic indicators.

The recent glacial retreat shows a good relationship with running means of Christchurch mean annual temperatures, allowing for a few years' lag in the response time of the glacier (Fig. 1). Hokitika precipitation has a high correlation with Franz Josef precipitation². There seems, however, to be little correlation with recent glacial movements. It seems that the west coast glaciers are not in equilibrium with the present warmer climatic regime and that they are responding by retreating rapidly. The better agreement with temperatures validates the use here of the position of the terminal face and past terminal moraines as indicators of palaeotemperatures.

A glacial advance will usually obliterate all terminal moraines in its path. The most advanced terminal moraines of the Franz Josef and Fox glaciers were laid down around 1600 AD. An advance of a greater magnitude occurred at the Franz Josef glacier at 4,730 yr BP. Since 1600 AD, a minor retreat occurred

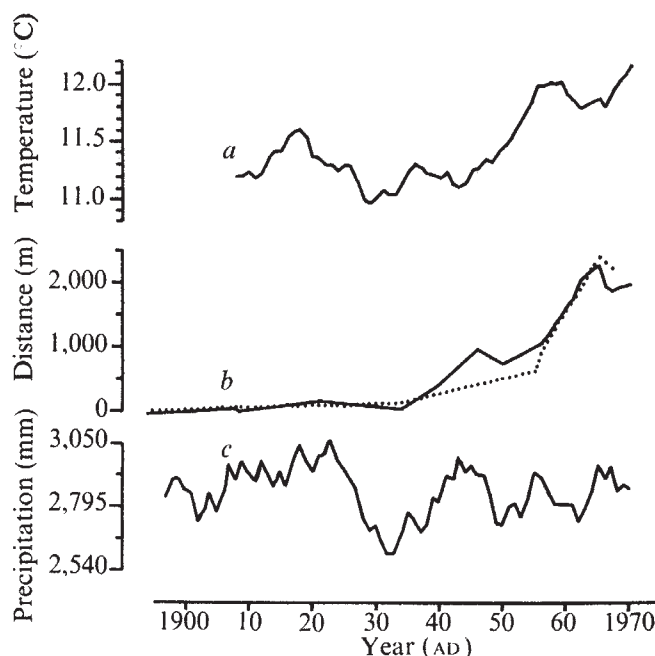


Fig. 1 Comparison of recent New Zealand west coast glacial behaviour with climatic parameters: *a*, seven-year running means of mean annual temperature at Christchurch; *b*, fluctuations of the terminal faces of the two west coast glaciers (—, Franz Josef; ··· Fox); *c*, seven-year running means of Hokitika annual precipitation.

up to 1800, after which the rate of retreat was moderate; since 1934 it has been rapid (Fig. 2 and ref. 3).

During the period 1600–1800, the glaciers were much more advanced than at present and were also apparently in equilibrium, which indicates a climate much cooler than at present. The shrinkage of the terminal faces since 1800 suggests a climatic amelioration, and the recent rapid wasting correlates well with the recent rapid climatic warming experienced in New Zealand.

Evidence of temperature changes before 1600 AD have to be gleaned from other palaeoindicators. Wardle notes a regeneration gap occurring in New Zealand gymnosperms between 1300 and 1900 AD, with the gap being most accentuated between 1600 and 1800 AD (ref. 4). Holloway, however, postulated a climatic deterioration around 1300 to explain anomalies

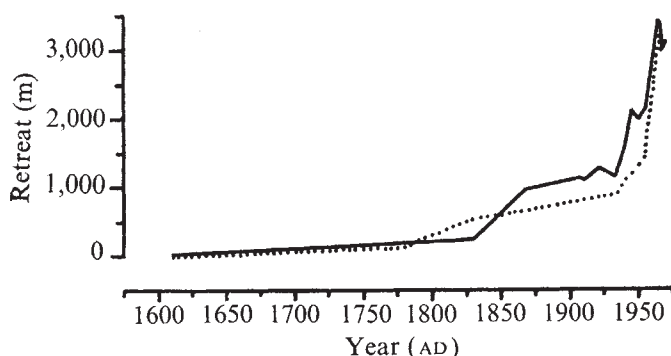


Fig. 2 Retreat of the terminal faces of the Franz Josef (—) and Fox (···) glaciers from their most advanced positions in the last millennium.

in the age structure of certain indigenous conifer stands on the South Island⁵. Molloy has suggested that cultural factors such as fire could have contributed to these anomalies⁶. Both Wardle and Holloway have examined forests in areas of dependable precipitation. With the coincidence between these ecological indicators and west coast glacial chronology⁷, non-climatic explanations seem untenable. Thus, it seems that New Zealand experienced a climatic deterioration starting around 1300 AD. Being most severe between 1600 and 1800, it correlates well with the Little Ice Age of the Northern Hemisphere⁸. Since 1800, a slow warming has occurred, culminating in the rapid, 1 °C warming of the past 40 yr.

New Zealand palaeotemperatures seem to be similar to those of Northern Hemisphere⁹ areas except during the present century, but some doubt must remain since dating was not accurate before temperature records were kept. Divergence between Northern Hemisphere and Southern Hemisphere temperatures may be a phenomenon of the past 50 yr. This assumes that New Zealand trends mimic those covering a wider area of the Southern Hemisphere. Evidence from south-eastern Australia (M. J. Coughlan, unpublished) Scott Base in Antarctica, Campbell Island, and Orcadas Island near South America at latitude 60°45'S, all demonstrate a temperature rise beginning in the 1940s.

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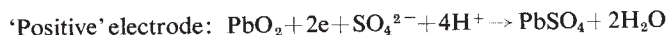
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Hydrodynamic instabilities in a lead acid battery

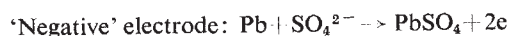
WE demonstrate here the feasibility of the observations of hydrodynamic instabilities in a lead acid battery during the discharge process. We use a system comprising two horizontal electrodes immersed in a transparent cell. The electrodes contain in the charged state porous PbO₂, the 'positive' electrode, and porous Pb, the 'negative' electrode. The electrodes were prepared to commercial standards using common industrial processes.

Hydrodynamic patterns in a transparent medium, with accompanying gradients of specific gravity, can be easily followed using a coherent and monochromatic light source. Specific gravity gradients in liquids are usually accompanied by parallel gradients in the index of refraction. Therefore, phase differences induced in the coherent light on passing through the medium are observed as an amplitude pattern when viewed out of the image plane. This is an interference phenomenon occurring between light waves traversing different optical paths in the medium.

The experiment was performed as follows. Light from a 5,145-Å Argon laser passes through a spatial filter and is then collimated on the Pb–PbO₂ transparent cell. The hydrodynamic pattern which occurs between two horizontal electrodes is projected on to a screen and recorded on photographic film. A few examples above a certain current density during the discharge process is predictable from stability considerations. The chemical reaction during the discharge process is

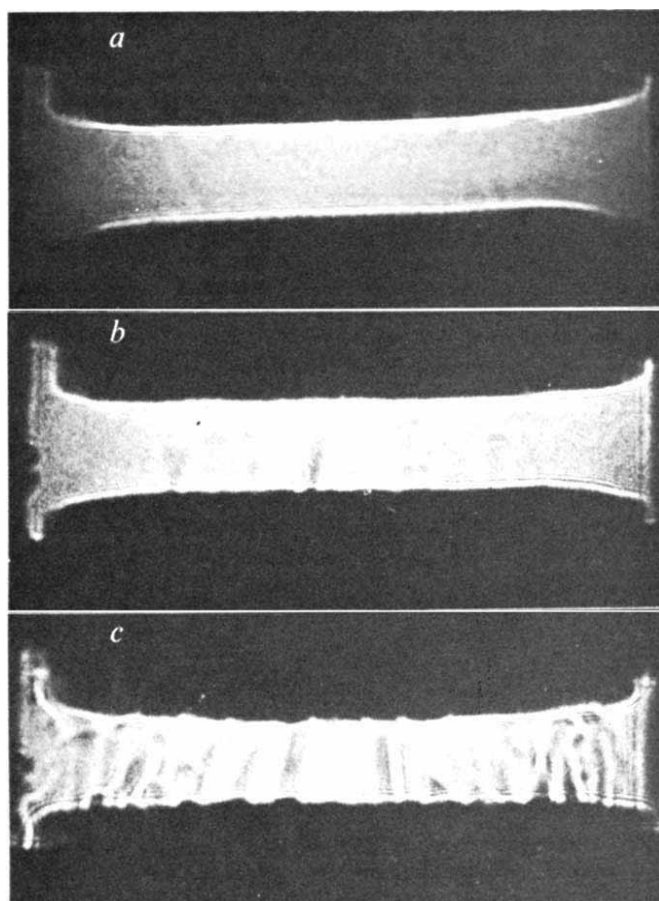


(Similar reactions occur with HSO₄[–] ions)



The electrodes therefore serve as a sink for the acid and the 'positive' electrode is a source of water. In a horizontal set-up of the electrodes at low enough currents, a steady-state profile of acid concentration, and, therefore, of density will be established. This profile is shown schematically in Fig. 2. To a first approximation a low acid concentration is established at the

Fig. 1 Hydrodynamic patterns between electrodes: a, $i = 0$ A cm^{–2}; b, $i = 6 \times 10^{-3}$ A cm^{–2}; c, $i = 30 \times 10^{-3}$ A cm^{–2}. All experiments performed at 25 °C.



electrodes' surfaces, and a maximum concentration is established somewhere in the electrode gap.

This distribution of density in the y direction can be sustained up to a certain gradient for a given cell geometry. The maximum current density which develops at the maximal gradient is easily calculated from the expression

$$i_{\max} = 2eD(\nabla C)_{\max}$$

where e is the elementary charge and D is the diffusion coefficient for H_2SO_4 aqueous solution. Thus, for example, with an electrode gap of 1 cm; ($y = 1$ cm) and a 1.2 g cm^{-3} acid (27% by weight), we have

$$\begin{aligned} (\nabla C)_{\max} &\simeq \frac{\text{Number of carriers}}{y_0/2} \\ &= \frac{(\text{Weight \% H}_2\text{SO}_4) \times (\text{Avogadro number})}{(\text{Molecular weight of H}_2\text{SO}_4) \times (y_0/2)} \\ &= 3.3 \times 10^{21} \text{ carriers per cm}^2 \\ D_{\text{H}_2\text{SO}_4} &= 1.34 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \end{aligned}$$

Therefore

$$i_{\max} = 14 \times 10^{-3} \text{ A cm}^{-2}$$

This estimation of $i_{\max}$ is really an upper limit since the y gradient of water generates a flow opposing the above current.

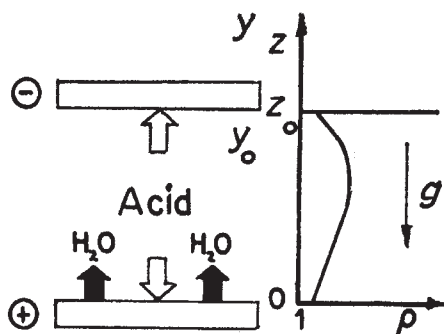


Fig. 2 Schematic profile of electrolyte density between electrodes at a steady state.

For current densities exceeding $i_{\max}$, as imposed by an external load, the y -direction diffusion processes of acid and of water are incapable of transferring the required flow rates of carriers, and an hydrodynamic instability can be expected. The development of this instability involves the creation of a water layer of low density at the bottom electrode, with a region of high density on top of it. In a gravitational field this inverted distribution of densities is discharged into patterns of ascending water and descending acid (Fig. 1).

Experimentally, an instability occurs at $\sim 5 \text{ mA cm}^{-2}$ and a pattern of pillars emanates from the bottom positive electrode towards the top negative electrode. The pillar pattern is of quite a regular geometrical nature and is preserved up to quite high current densities of $\sim 0.5 \text{ A cm}^{-2}$. Only above that limit does the pattern break so that turbulent phenomena are observed.

We believe that the hydrodynamic instability described, and its observation, may provide a useful tool for the understanding of the performance of Pb-PbO₂ batteries at high discharge currents. The system may serve as a model to elucidate the limits imposed by hydrodynamic phenomena on the performance of the lead acid battery. It should be pointed out that

the phenomena presented occur in the whole range of technically feasible discharge rates.

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Tungsten trioxide as a photoanode for a photoelectrochemical cell (PEC)

THE PEC¹ has acquired popularity among researchers in the past years, mainly because of its possible use in solar energy conversion^{2,3}. The major problem at present is to find a semiconductor electrode with an optical band gap (written as just band gap below) small enough to allow it to absorb a reasonably large portion of the solar spectrum, and at the same time being stable against photocorrosion. We here report that WO₃ meets these criteria although it is not very efficient at solar wavelengths.

Until now, the only material which was known to be stable as a photoanode absorbing in some region of the solar spectrum, is TiO₂, with a band gap $E_G = 3.05 \text{ eV}$ corresponding to a wavelength of 408 nm—on the border of the visible and ultraviolet. In the search for other stable photoanodes of smaller E_G , one criterion, which we used, is that one of the chemical elements making up the semiconductor material should be capable of reversibly changing its valence state to accommodate a hole without decomposing the semiconductor (as $\text{Ti}^{3+} \rightarrow \text{Ti}^{4+}$ in non-stoichiometric TiO₂) instead of having only one possible stable valence state in the semiconductor (as Cd^{2+} in CdS or Zn^{2+} in ZnO, both of which are decomposed by holes). WO₃, an n-type semiconductor with $E_G = 2.8 \text{ eV}$, satisfies this criterion, and was selected for trial as a photoanode in a PEC.

WO₃ electrodes were prepared either by heating tungsten metal to form a layer of yellow WO₃, or by spraying ammonium tungstate on conducting glass (a gold layer on glass) and heating at $\sim 500^\circ\text{C}$ to decompose the tungstate. Higher photocurrents were obtained from electrodes formed by heating the metal, but apart from the spectral response of the photocurrent, the general properties were much the same for both types of electrodes. X-ray powder diffraction of the samples showed the composition of the layers to be WO₃ with traces of lower oxides. The WO₃ electrode was connected to a platinised platinum oxygen counter electrode, and both electrodes were immersed in 1 N H₂SO₄ in a quartz cell.

In AM1 sunlight (AM1 = airmass 1, which is equivalent to directly overhead sunlight with energy of $\sim 1 \text{ kW m}^{-2}$) photocurrents of $\sim 0.2 \text{ mA cm}^{-2}$ were obtained with WO₃ on tungsten and typically, one quarter of this for WO₃ on conducting glass. (Dark currents were $< 10^{-4} \text{ mA cm}^{-2}$.) The open circuit potential between the two electrodes increased from $\sim 280 \text{ mV}$ in the dark to 400 mV on illumination. Figure 1 shows the difference in currents obtained, in a potentiostatic experiment, between a WO₃ electrode under illumination and in the dark. The start of oxygen evolution shifted from $+2.0 \text{ V}$ (an overpotential of $\sim 0.8 \text{ V}$) in the dark to $+0.6 \text{ V}$ (an underpotential of $\sim 0.6 \text{ V}$) on illumination—a shift of 1.4 V .

That the photocurrent came from oxidation of water, and not photocorrosion of the WO₃ layer, is evident from two observations. First, after continuous passage of 42 C of electricity, which, if 100 per cent corrosion occurred, would correspond

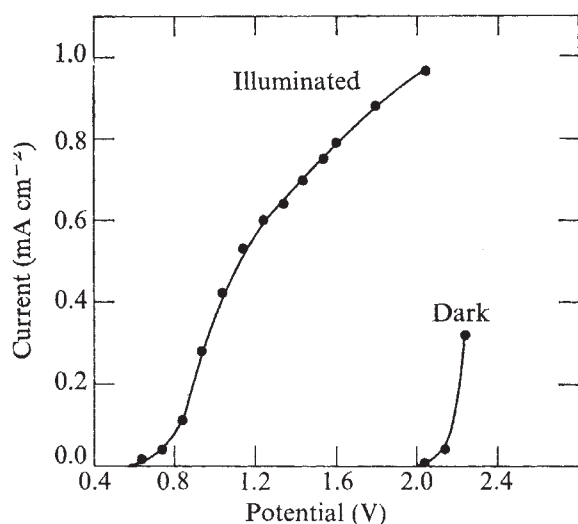
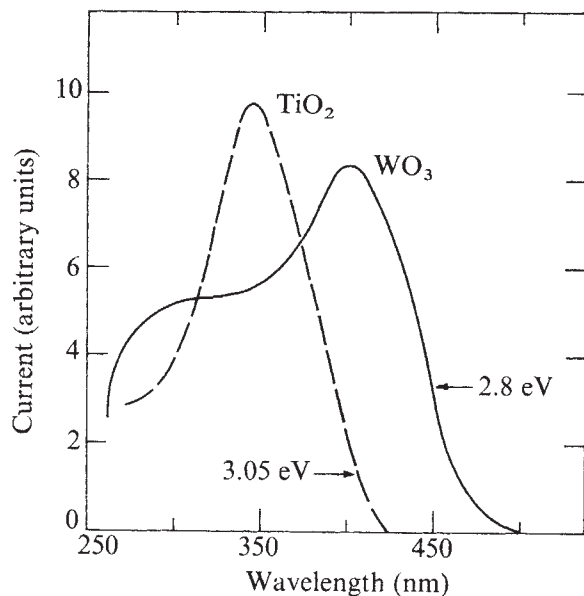


Fig. 1 Current-potential curves for a WO_3 -tungsten photoanode in the dark and on illumination with light approximating to sunlight. (Measurements were made using a standard calomel electrode (SCE) as reference, and the potentials shown are adjusted to the standard hydrogen electrode (SHE) scale.)

to the dissolution of 50 mg of WO_3 (assuming the hypothetical dissolution occurred by a two-electron transfer), there was no change in current, and no change in the weight of the electrode to < 0.5 mg. Second, if a strong source of light was used, so that a larger current ($\geq 1 \text{ mA cm}^{-2}$), was obtained, gas evolution from the electrode was clearly evident.

Besides stability, the next important property of a suitable photoanode for solar energy conversion is the ability to absorb well in the visible spectrum. Theoretically, the absorption edge for WO_3 should be shifted ~ 40 nm to the red, compared with TiO_2 . The spectral response of the WO_3 electrode is compared in Fig. 2 with that for a polycrystalline TiO_2 electrode prepared by heating titanium metal in oxygen, followed by heating in hydrogen.

Fig. 2 The spectral response of the photocurrent for an electrode of WO_3 on conducting glass compared with a polycrystalline TiO_2 electrode. The positions of the wavelengths corresponding to the band gaps are arrowed ($3.05 \text{ eV} \approx 410 \text{ nm}$; $2.8 \text{ eV} \approx 450 \text{ nm}$). The current at the peak for TiO_2 is actually considerably higher than the corresponding current for WO_3 —they are shown nearly equal for purposes of clarity. (The total short-circuit current in sunlight for the TiO_2 electrode is $\sim 0.8 \text{ mA cm}^{-2}$ compared with $\sim 0.2 \text{ mA cm}^{-2}$ for a WO_3 electrode.)



The absorption edge for WO_3 was, in fact, shifted by ~ 50 nm. Figure 2 shows typical results obtained with WO_3 on glass. For WO_3 on tungsten, instead of the current decreasing with decreasing wavelength after the peak at 400 nm, there is a broad plateau, or slow increase, between 400 and 360 nm, followed by a sharper increase to a peak at ~ 300 nm—much like the mirror image of the curve shown. It should be stressed that the part of the curve in the visible region, 400–500 nm, is similar for both types of electrode. The difference in the ultraviolet may conceivably arise from a difference in crystallinity of the two types of WO_3 . In other materials such as Si, there is a difference in spectral response between the amorphous and the crystalline states².

The important point arising from these preliminary results is not to suggest that WO_3 may be a serious candidate for an economical solar cell (the absorption still occurs over too small a part of the solar spectrum) but rather to show that stability against photocorrosion is not unique to TiO_2 (or other semiconductors with an even larger gap, such as Ta_2O_5 , which may also be stable, but do not absorb in the solar spectrum), and that, where one more stable compound with a smaller band gap has been found, there may be others with even smaller gaps.

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Effect of surface roughness on rolling friction and adhesion between elastic solids

THEORETICAL analyses of the influence of surface roughness on the adhesion of elastic solids^{1,2} suggest that the introduction of roughness should reduce the adhesion to an extent governed by an adhesion parameter

$$\alpha = \left(\frac{4\sigma}{3} \right) \left[\frac{4E}{3\pi\beta^2\gamma} \right]^{2/3}$$

where α is the distribution of asperity heights (assumed to be Gaussian), β the mean radius of curvature of the asperities and γ is the net change in surface energy per unit area on the formation of adhesive contact. Experiment³ bears out the theory. Here we report experiments on rolling, when we found α again to be a useful parameter. At low roughnesses, however, we found a surprising new result, that the adhesion could be greater than for smooth surfaces, and we propose an explanation for this.

Roberts and Thomas³ and, independently, Kendall⁴ had shown that in rolling friction the adhesion term may sometimes be dominant. In these circumstances the rolling friction provides a direct measure of the work required to peel apart and re-adhere the interface. This depends on the velocity, and may be thought of as the product of the surface energy and a viscoelastic term.

For smooth surfaces, the force required to separate a sphere from a flat had been shown by Johnson *et al.*⁵ to be

$$P = -(3/2)\gamma\pi R$$

The reduction in the force with increasing roughness comes from the high asperities prising the surfaces apart and reducing the true area of contact. It is therefore to be expected that

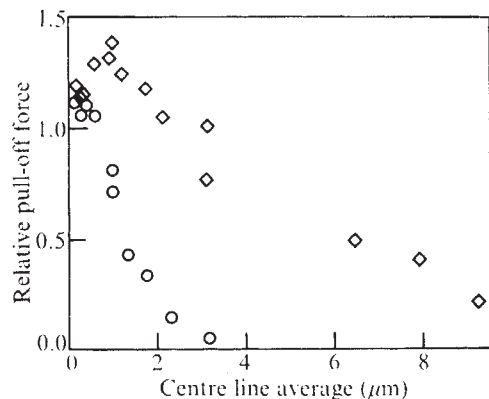


Fig. 1 Relative pull-off force for a smooth rubber surface in contact with a Perspex surface as a function of the roughness (centre line average) of the Perspex, using a crossed cylinders configuration. The pull-off force is normalised to be unity for a nominally smooth Perspex surface. $\circ$, RTV 602 ($E = 4.9 \times 10^8$ Pa); $\diamond$, Dow Corning XF-13-523 ($E = 6.3 \times 10^4$ Pa). In both cases the pull-off force for a smooth surface indicated a surface energy of $\gamma \approx 100 \text{ mJ m}^{-2}$. For a Gaussian distribution of asperity heights, the centre line average is a good measure of σ .

adhesion measured by rolling experiments will be affected by surface roughness in a similar way to the pull-off measurements.

We measured the adhesion during rolling at various speeds, for two rubbers, over a range of roughnesses. Perspex cylinders were roughened by blasting with fine particles. The surfaces were characterised by profilometry measurements, with data processing using established techniques⁶ and thus β and σ were deduced. These cylinders were rolled down an inclined plane surface of silicone rubber and the time taken to roll 140 mm was measured electronically. The adhesion energy was determined from the weight of the cylinder and the angle of inclination. These measurements were much more reproducible ($\pm 2\%$) than those of Fuller and Tabor, partly because the adhesion was averaged over the whole of the surface of the cylinder and also because the time dependence was precisely specified. The greatest errors were in the surface characterisation ($\pm 10\%$), also depending on sampling lengths.

For the purpose of comparison with previous work the pull-off force for the various surfaces was measured using a crossed cylinders configuration. The results are shown in Fig. 1. Figure 2 shows the results for the rolling experiment, and a comparison of these two graphs shows that the effects of changing roughness and modulus are very similar in the pull-off

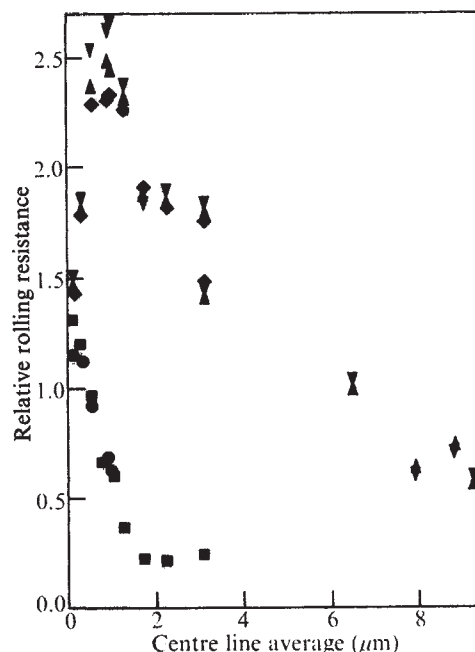


Fig. 2 Relative rolling resistance for Perspex cylinders (radius 5 mm, length 10 mm) rolling down an inclined smooth rubber surface at the following speeds (mm s^{-1}). RTV 602: $\bullet$, 2; $\blacksquare$, 4; $\blacktriangle$, 6; Dow Corning XF-13-523: $\blacklozenge$, 0.5; $\blacktriangle$, 1; $\blacktriangledown$, 2. The adhesion energy is the net work required for a complete cycle of adhering together and then peeling apart unit area of interface, and is deduced directly from the rolling resistance. It is markedly velocity dependent^(3,4,7). The absolute values for nominally smooth surfaces, to which the above points are normalised, are respectively (mJ m^{-2}): 600; 970; 253; 384; 612. Dusting the rubber surface with talcum powder to remove the adhesion reduced the relative rolling resistance to a very small value, indicating that bulk ploughing hysteretic losses were almost negligible in these experiments.

and rolling experiments. In the case of the rolling experiments, it seems possible (except for the peak discussed below) to separate out the viscoelastic time dependence and the effect of roughness, since the normalised points for different rolling speeds fit closely over one another when the normalised adhesion ≈ 1 .

A new feature became apparent during these studies, however, which had previously been neither predicted nor observed. For very small roughnesses, the adhesion may be considerably

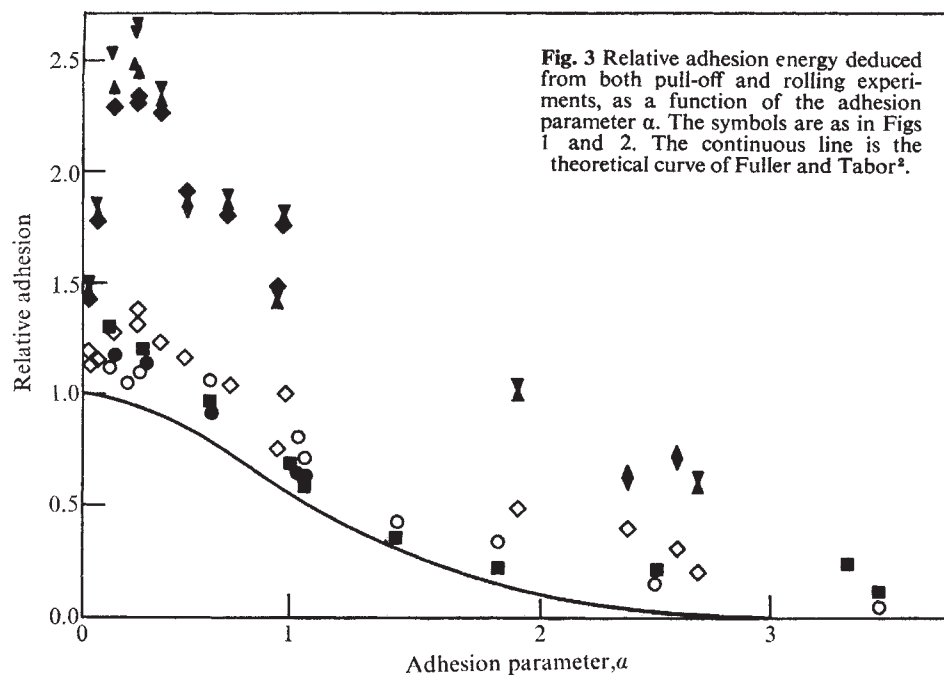


Fig. 3 Relative adhesion energy deduced from both pull-off and rolling experiments, as a function of the adhesion parameter a . The symbols are as in Figs 1 and 2. The continuous line is the theoretical curve of Fuller and Tabor².

greater than the adhesion with a nominally smooth surface. This effect is greatest when the effective surface energy is large and the modulus is small, and it is especially marked in the case of rolling on soft rubber. These results are shown in Fig. 3, where adhesion (normalised to the adhesion against a nominally smooth surface under the given conditions) is plotted against the adhesion parameter α . The theoretical curve of Fuller and Tabor is shown by the continuous line. The adhesion parameter seems to be a useful way of describing the influence of topography on the normalised adhesion, all the curves falling to half their maximum value when $\alpha \approx 1$.

The increase of the adhesion for small roughnesses may be accounted for as follows: when a hard sphere is separated away from an elastic surface, there exists a minimum stable contact area. At this point the stored elastic energy is 1.4 times greater than the surface energy of the contact area. If the separation is increased quasi-statically, the rubber pulls away completely from the spherical surface, and all this elastic energy is lost. If the rate of separation is increased, then, because of viscoelastic effects, the elastic energy lost may be greater still.

For almost smooth surfaces, most of the contact areas between asperities and the rubber are smaller than the critical value and the surfaces therefore peel apart in a similar manner to perfectly smooth surfaces. For very larger roughnesses, the reduction in the true area of contact dominates. But between these two extremes a substantial proportion of the asperities may have a contact area just greater than the critical value, and therefore an increase in adhesion of up to 40% is possible. Simple analysis indicates that α should be an adequate parameter to determine the roughness at which this effect is important with the peak at $\alpha \approx 0.2-0.3$. Comparison of these predictions with Fig. 3 is very satisfactory.

It will be noted that there is an exceptionally large increase in normalised adhesion in the case of the rolling experiments on very soft rubber. This may be due partly to the viscoelastic effect described above, and also to a snowballing of the adhesion (the extra adhesion at the rear of a rolling cylinder pushes more asperities into the rubber at the front, which increases the adhesion and so on).

A detailed account of the theory is in preparation. We wish to thank Professor D. Tabor for advice and encouragement and also Dr Whitehouse and Mr D. Kinsey of Rank Taylor Hobson for their help with the surface topography measurements. We are grateful to the SRC for an ASSIST award to G.A.D.B.

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approach, which has proved useful in understanding time-dependent phenomena (see refs 4 and 5).

Pitting corrosion can be regarded as the failure of a two-dimensional film, which is altered over a finite time. Thus a stochastic approach is needed to explain many of the unsolved problems of pitting corrosion, such as the size effect and sweep rate sensitivity of pitting potential, and the relationship between pitting potential and induction time.

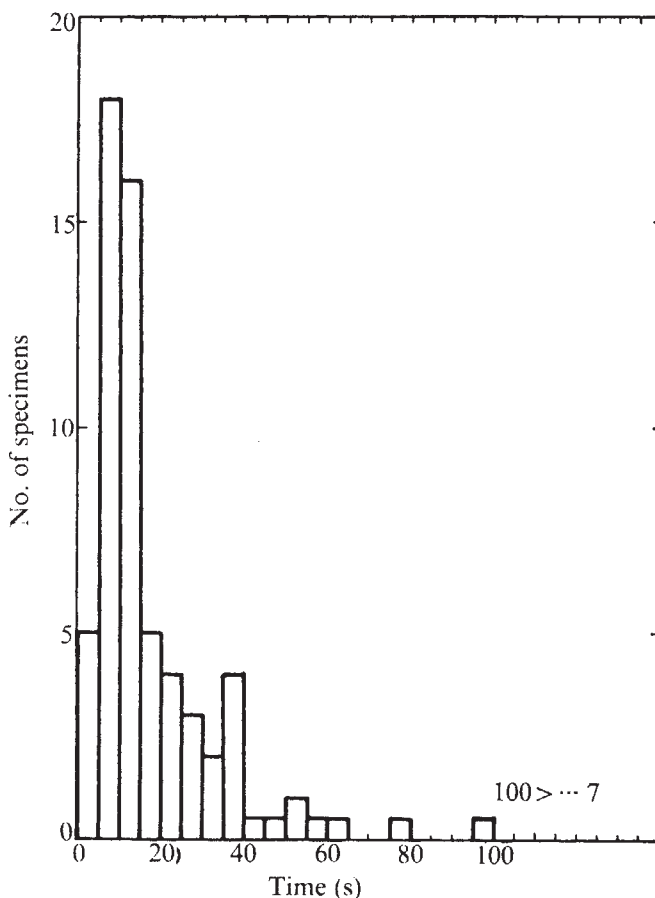
The stochastic approach requires the measurement of several series of data taken during the pitting process, in the same controlled conditions. We have developed a multichannel pitting corrosion tester which can measure the pitting potential or induction time of 12 specimens consecutively, using one potentiostat. Figure 1 is a histogram of induction time for 72 specimens of 18-8 stainless steel, the potential of which was kept constant at 0.35 V in 3.5% NaCl solution saturated with nitrogen gas at 35 °C. Though further experiments revealed that variations in pitting potential obey the normal distribution a distorted type of distribution was observed for the induction time.

This behaviour can be explained using the stochastic theory developed⁴ for the brittle fracture of glass. The transition probability, $\lambda(t)$, of pit formation at any time, t , after applying the potential, is given by

$$\lambda(t)dt = -dP(t)/P(t) = -d \ln P(t) \quad (1)$$

where $P(t)$ is the survival probability, that is, the number of

Fig. 1 Histogram of induction time for pitting at constant potential (0.35 V) for 304 stainless steel in 3.5% NaCl solution saturated with nitrogen gas at 35 °C (72 specimens). Surface of specimens was polished with 6/0 emery paper before immersion into solution. After holding specimens in natural corrosion state for 5 min, and for further 5 min at -0.10 V to give a constant surface condition, the potential of the specimens was shifted to +0.35 V, at which potential pitting occurred after the induction time.



Pitting corrosion as a stochastic process

We believe that a stochastic theory originally developed to explain the fracture of solid materials under stress can be used to explain pitting corrosion. In the past (see ref. 1), both pitting potential² and induction time³ have been used to select resistant alloys and to search for possible breakdown mechanisms. Studies of these and other phenomena, however, always reveal a wide scatter of data, so we have used the stochastic

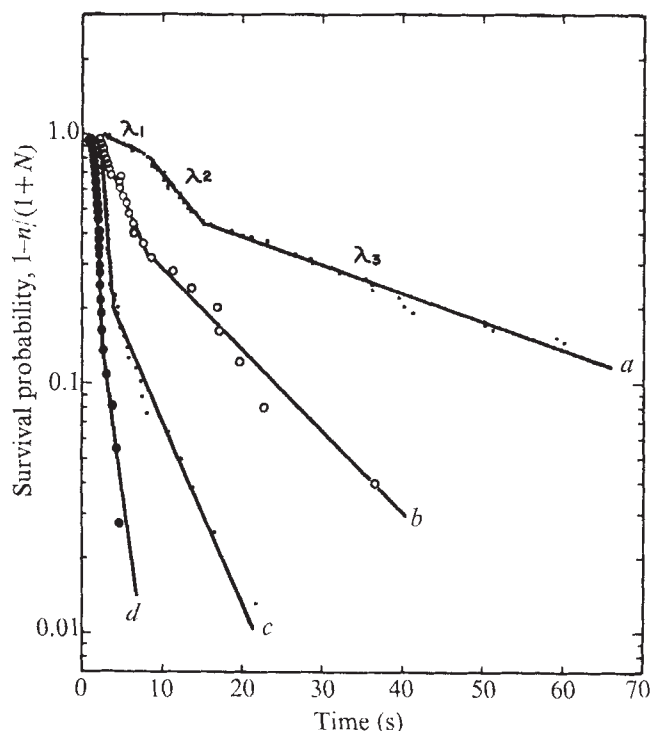


Fig. 2 Change in survival probability with time *a*, 0.35 V; *b*, 0.36 V; *c*, 0.37 V; *d*, 0.378 V. The slope of $\log P$ against time gives transition probability.

non-pitted specimens at time t . This equation is integrated for $P(0) = 1$, giving

$$P(t) = \exp\left(-\int_0^t \lambda(t) dt\right) \quad (2)$$

Generally, $\lambda(t)$ is a function of time, but if it is not, equation (2) can be transformed to

$$\ln P(t) = -\lambda t \quad (3)$$

Equation (3) predicts a linear relationship between the logarithm of survival probability and the time elapsed after applying the potential. The survival probability is defined as $1 - n/(1 + N)$, where N is the total number of specimens and n is the number of specimens failed in time t . Figure 2 shows such a plot for various potentials; the straight line which is divided into three. The slope of the lines gives the transition probability, as understood from equation (3).

The three distinct divisions could correspond to three processes causing pit formation at any one potential. The first process has the lower transition probability of λ_1 , compared with λ_2 for the second process, with almost no delay. The second process begins only after a delay, in spite of the higher transition probability, λ_2 . The third process has the lowest transition probability of λ_3 , as shown in Fig. 2. All are highly dependent on potential, and λ_1 in the first process is described by

$$\begin{aligned} \lambda_1 &= \alpha_1(E - E_{crit}) \\ &= 8.20(E - 0.346) \end{aligned} \quad (4)$$

where E is the potential applied to the specimen in volts, and E_{crit} is the critical potential, at which no pitting is expected. It is interesting to note that the same linear dependence of induction time on potential has been observed by Sato *et al.*⁶. Both λ_2 and λ_3 are described by the exponential function of potential.

When transition probability is a function of potential, the

most probable value of the pitting potential can be obtained using the equation⁶

$$v d\lambda/dE|_{E=\bar{E}} = \lambda^2 \quad (5)$$

which is derived by differentiation of the probability density function of pitting potential, where v is the sweep rate of potential and $\bar{E}$ is the most probable value or mean value of pitting potential. If the first process determines the pitting potential, the sweep rate sensitivity of pitting potential can be obtained by the substitution of λ_1 into λ in equation (5)

$$\begin{aligned} \bar{E} &= (v/\alpha_1)^{1/2} + E_{crit} \\ &= 0.349v^{1/2} + 0.346 \end{aligned} \quad (6)$$

This predicted relationship has been confirmed in another set of experiments for determining E with changing potential sweep rates. This indicates that only the first process operates to cause pitting potential. It should also be emphasised that no paper has ever mentioned such dependence of pitting potential on the square root of the potential sweep rate. This relationship could be used to determine the value of the critical potential by extrapolating to the zero sweep rate even if the potential sweep method is used. The stochastic theory also predicts that the lowest pitting potential can be expected on the largest area of specimen. This could explain the unexpected failures which often occur in practical large scale apparatus in spite of proved resistances in laboratory tests using specimens with small areas.

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Primary production of oxygen from irradiated water as an explanation for decreased radiobiological oxygen enhancement at high LET

WE report here the results of sensitive measurements of oxygen yields in water irradiated with radiation of high linear energy transfer (LET), in conditions which precluded the formation of oxygen in secondary reactions. These yields are important in radiobiology because, although small, they are sufficient to account for the decrease in value of the oxygen enhancement ratio found for biological cells irradiated by radiation of high LET compared with the value for low LET radiation.

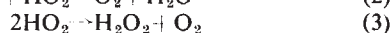
An important feature of radiation damage to biological cells is that their sensitivity is greater in the presence of oxygen than under anoxic conditions^{1,2}. For a given type of radiation the ratio of the two sensitivities, known as the oxygen enhancement ratio (OER) increases with increasing oxygen concentration. With radiation of increased LET, the anoxic sensitivity to radiation damage is increased, but the OER for a given oxygen concentration is decreased. Among the hypotheses advanced to explain these phenomena either independently, or as parts of a single mechanism, is the suggestion that the reason for decreased values of OER at high LET is the presence of oxygen

Table 1 *G* values of primary HO₂ and O₂

Ion or radiation	⁶⁰ Coγ	⁴ He	⁴ He	¹⁴ N	¹⁴ N	¹⁴ N	¹⁴ N	¹⁴ N	²⁰ Ne
Energy (MeV)	1.25	29	6	127	87	45	38	34	30
<i>G</i> (HO ₂)	0.03	0.075	0.08	0.11	0.12	0.20	0.22	0.25	0.25
<i>G</i> (O ₂)	0	0.0016	0.008	0.007	0.0085	0.012	0.012	—	0.031

formed in, or close to, the track of the ionising particle³. As a result, the radiobiological sensitivity is already raised above the hypothetical anoxic level, making further addition of oxygen less effective than with radiation of low LET, for which oxygen is not a radiolytic product. Alper and Bryant² have shown that the 'oxygen-in-the-track' hypothesis is superior to other hypotheses, for example, the suggestion that radicals formed in the track may recombine if sufficiently close together, thereby fixing damage without involving oxygen⁴. Experiments based on the oxygen-in-the-track hypothesis have been interpreted to quantify, by inference, an effective concentration of the oxygen present in cells along the track of energetic ⁴He ions at the time of radiobiological action. Our measurements now provide direct radiation-chemical evidence that oxygen is formed as a primary product in sufficient yield to account for the decrease in OER at high LET.

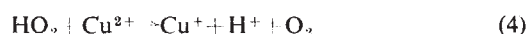
Oxygen can be formed both with low and high LET radiation by the familiar bulk secondary reactions⁵ of the normally accepted radiolytic products (OH, HO₂, H₂O₂, OH⁻, H⁺, H, H₂, e_{aq}⁻) such as



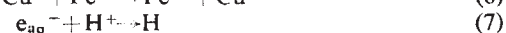
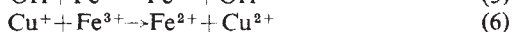
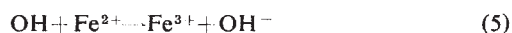
These secondary reactions are, however, suppressed by low concentrations of solutes, or are negligible in the time scale of spur and track reactions ($\leq 10^{-8}$ s) so that even reaction (1) cannot be invoked as an explanation for the formation of HO₂ in the track^{6,7} because of its low rate constant.

Our method of preventing the occurrence of such secondary reactions and of measuring reproducibly the small primary yields of oxygen was as follows. Argon was bubbled through a suitable aqueous solution, irradiated by heavy ions from the Harwell Variable Energy Cyclotron at low currents (~ 2 nA) with adequate stirring, and then through a Hersch cell⁸, the current from which gives a measure of the oxygen concentration in its own electrolyte. The area above the baseline of the graph of Hersch cell current against time obtained with constant argon flow provides a quantity proportional to the amount of oxygen formed during irradiation, which was converted to an absolute quantity by calibration with an electrolytic cell, also in the gas circuit. The methods of particle charge and energy measurement needed to obtain the yield per 100 eV, the *G* value, have been described⁹. By the use of methods and various solutes (unpublished work), measurements of oxygen yields have been used to obtain primary *G* values of HO₂, O₂, H₂O₂ and H₂ for γ rays, and some of these for several different kinds of radiation.

The principle of the method to measure HO₂ is to generate oxygen by the reaction of HO₂ with cupric ion^{10,11}

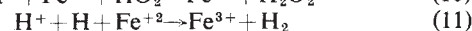
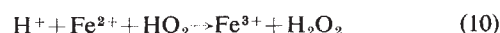


in a deaerated solution of ferrous ammonium sulphate (10^{-3} M) and cupric sulphate (10^{-2} M) adjusted to pH=2 with sulphuric acid. Other relevant reactions are



Any primary oxygen yield will also be measured from this solution and so must be subtracted from the overall oxygen yield.

The yield of O₂ was obtained as the lowest limiting yield when the concentration of Fe²⁺ ions was increased in deaerated Fricke solutions (ferrous ammonium sulphate 10^{-3} M, sulphuric acid 0.4 M), and was usually obtained between 10^{-2} and 10^{-1} M Fe²⁺. The relevant overall reactions^{12,13} are (5), (7), (9), (10) and (11)



Some results are shown in Table 1.

The mean instantaneous concentration of a track product c_0 mol l⁻¹ can be derived from the initial track *G* value *G*₀, the mean LET, *L* (eV nm⁻¹) and the mean track radius *r*₀ (nm): $c_0 = G_0 L / 60\pi r_0^2$. In the absence of further reaction c_0 will change with time to give c_t dependent on time

$$c_t = G_0 L / 60\pi(r_0^2 + 4Dt)$$

where *t* is the time (s) and *D* (nm² s⁻¹) is the diffusion constant. For typical α-particle values¹⁴ of *L* and *r*₀, 10^3 and 1 respectively, c_0 for oxygen would be 4.2 mM and after 10^{-8} s, c_t would be 51 μM (taking $D = 2.1 \times 10^9$ nm² s⁻¹ (see ref. 15)) if *G*₀ were assumed to be the measured value. If, however, oxygen were generated in the track core, the diffusion theory suggests¹⁶ that much of it would be converted to O₂⁻ and HO₂ by track reactions (12) and (13)



possibly giving rise to the yield of HO₂ measured in acid solution. Initial track core concentrations of oxygen may therefore be higher than c_0 given above, and this is being investigated.

The way in which oxygen increases radiation sensitivity may be by reaction with lesions in the biological material formed by direct (or indirect) action in the δ-ray sheath surrounding the track core. For α particles of 5 MeV (90 eV nm⁻¹) Alper and Bryant find an OER for survival of 1.5 for two biological systems, *Chlamydomonas* and *Shigella*², for which the OER for γ-ray survival is 2.5. Using a mechanism based on competition between repair and fixation derived from data for low LET radiation they estimate an effective stationary oxygen concentration in the irradiated track volume of 2.2 μM. By interpolation of the results in Table 1 for 29 MeV and 6 MeV α particles (which are based on the average LET for the whole track) it can be shown that the *G* value for oxygen is ~ 0.005 . The concentration of 2.2 μM together with this *G* value implies an irradiated track volume with a radius of 33 nm, in which it can be shown¹⁷ $\sim 90\%$ of the dose is initially deposited. This indicates that the small yield of oxygen is sufficient to produce the observed effect.

The above argument has been simplified in that variations of oxygen concentration with time, and therefore of its reaction rate, have been ignored. More detailed calculations¹⁸, however, including diffusion, using the same parameters for initial track geometry and the repair fixation competition confirm the adequacy of the oxygen yield. In addition, the reaction times are within those required¹⁸ for the oxygen effect. Work in the determination of oxygen and HO₂ yields at varying Cu²⁺ and Fe²⁺ concentrations, and on oxygen enhancement ratios for radiation types with similar LET but differing secondary electron spectra, is in progress to test conclusions.

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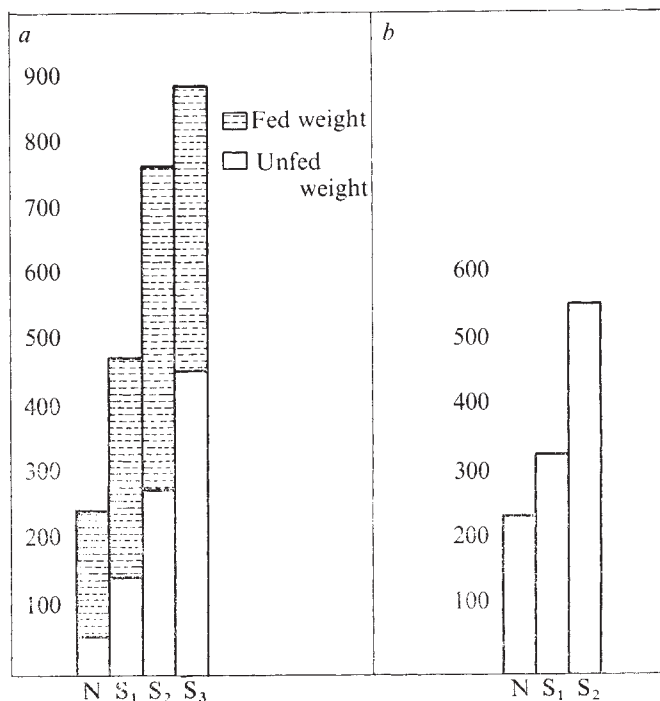


Fig. 2 The effects of ecdysone on body weight (a) and egg output (b) of female *Ornithodoros moubata* (w) super-moulted once (S₁), twice (S₂) and three times (S₃). Shaded areas, fed weight; unshaded areas, unfed weight (in mg).

Ecdysone and the super tick

Ticks and insects develop and grow through intermittent replacement of their integument. This moulting does not occur beyond the adult stage, with the exception of primitive apterygote insects such as silverfish, which continue to moult and grow after they become sexually mature. Moulting ceases to occur in the pterygote adult insect because the glands secreting ecdysone, the hormone required to induce moulting, break down and disappear soon after the insect (and presumably also the tick) becomes adult¹. The adult tissues of both hemimetabolous insects and those with complete metamorphosis continue to respond to ecdysone if exposed to it. There are, however, differences in the responsiveness of different segments of the insects and the resulting 'super-moulted' adult insects are usually defective²⁻³. We have found that ticks also retain the full potential to moult, grow and reproduce normally as adults if they receive ecdysone. Through successive adult moults 'super ticks' are obtained (Fig. 1) which consume very large volumes of blood and produce many more viable eggs than normal ticks.

Fig. 1 From the left, a normal fully engorged female compared with females super-moulted once, twice and three times.



We used the eyeless tampan, *Ornithodoros moubata* (Murray) (Argasidae), which is widely distributed throughout East Africa. We used adult ticks which were at least 1 month past their last moult. Females of this age are usually ready to take a blood meal and mate, and then lay about 250 eggs. Subsequent meals are necessary for additional batches of eggs. Ticks were divided into groups of 30 and fed on defibrinated pig blood through bat's membrane⁶. β -Ecdysone or the phytoecdysone ponasterone A were dissolved in 95% ethanol and added to the blood meal in concentrations of 1–5 $\mu\text{g ml}^{-1}$, the range which induces moulting in *O. moubata*⁷. After moulting, the ticks were offered a blood meal free of ecdysone, and then mated to assess their reproductive capacity. Another group was allowed to feed and moult as in the first group, and then fed again on blood containing ecdysone. The procedure was repeated again with the ticks that moulted on the second blood meal.

As Table 1 shows, if the blood contained ecdysone in a concentration greater than 2 $\mu\text{g ml}^{-1}$, most of the females moulted. With 4–5 $\mu\text{g ml}^{-1}$ many of the moulted females underwent a second moult—without a further blood meal. Thus it seems that ingested ecdysone is not destroyed during digestion in ticks, and continues to stimulate moulting when the contents of the gut are being absorbed. This effect is dose dependent and seems to be rather rare at the lower concentrations.

Adult males seem more refractory than females to induction of moulting by ecdysone. Many of them, however, secreted new cuticle that was only loosely attached and could be peeled away easily. These males always died. Ponasterone A, which is more active than β -ecdysone when fed to insects⁸, was more effective on ticks too, and at a dose of 5 $\mu\text{g ml}^{-1}$ was highly toxic.

If females were mated and then fed on blood containing ecdysone, they moulted first, and if this process did not completely drain the food reserve, the remainder was utilised for egg production. Thus in one experiment 50 inseminated females were fed blood containing β -ecdysone

Table 1 Effect of β -ecdysone and ponasterone A on moulting of adult *Ornithodoros moubata*

Dosage (μ g per ml blood)	No. super-moulted once	No. super-moulted twice*	No. of unsuccessful moults (in the first or second moult)	Overall death from treatment
β-ecdysone				
0 { M	0	0	0	0
0 { F	0	0	0	0
1 { M	4	0	9	10
1 { F	4	0	1	9
2 { M	3	0	17	17
2 { F	21	2	3	3
3 { M	3	0	15	15
3 { F	26	5	5	7
4 { M	14	0	14	15
4 { F	29	15	4	4
5 { M	17	0	11	20
5 { F	27	15	20	12
Ponasterone A				
0 { M	0	0	0	0
0 { F	0	0	0	1
2 { M	10	0	0	6
2 { F	19	0	0	1
3 { M	4	0	10	10
3 { F	25	0	4	5
4 { M	3	0	23	27
4 { F	19	0	8	10
5 { M	8	0	13	20
5 { F	4	0	7	26

Each group contains 30 ticks.

*Moulting twice without a second blood meal.

(5 μ g ml⁻¹). Twelve died and 38 moulted. Six out of the 38 then laid some eggs.

When super-moulted females were mated and fed blood, they consumed a much larger meal and consequently laid many more eggs than the normal tick. Blood utilisation seemed to be similar in normal and super-moulted ticks whether they moulted once or twice: for each 1 mg of blood consumed one egg was deposited (Fig. 2).

After one moult ticks were fed again on blood containing ecdysone (3 μ g ml⁻¹); 57 out of 95 moulted after that meal. These twice-moulted ticks were about twice as heavy as the once-moulted adults, and about five times heavier than normal adult females. They consumed about 500 mg of blood and laid about 500 viable eggs. The larvae that hatched from these eggs developed normally. Only about four out of 12 females moulted after a third blood meal containing ecdysone. At this stage the weight of the unfed tick was nine times that of the normal tick (Fig. 2).

Our results may have some practical consequences as the use of ecdysone and its analogues has been proposed as a means of controlling ticks¹⁰. Obviously 'super ticks', produced by the application of ecdysone or its analogues, would constitute a danger of increasing the damage done by this pest rather than controlling it.

Ponasterone A belongs to a group of ecdysones widespread in large concentrations in plants. Many of them act in lower concentrations than does insect ecdysone⁸, and it is conceivable that host animals might feed on plants containing large amounts of these substances, thus maintaining in their blood sufficient concentrations to induce super-moulted ticks. We have collected mature adult ticks from warthog burrows in the Nairobi National Park that were much bigger than ticks from the same source bred in the laboratory on pig blood. The possibility that warthogs feed on plants containing these phytoecdysones merits investigation.

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Transitory hemizyosity of paternally derived alleles in hybrid trout embryos

It is generally assumed that in species with yolk-rich eggs early embryonic development is sustained by maternally transmitted storage substances in the egg cytoplasm^{1,2}, while embryonic genes become activated later in development. As a rule, the maternally and paternally derived alleles of a particular gene locus start to function simultaneously at a defined developmental stage. But preferential activation of the maternally derived allele has been observed³⁻⁷. This prompted the suggestion^{2,4} that the information required to switch on embryonic structural gene loci is transcribed by the diploid nucleus of the mother during oogenesis. According to this hypothesis, regulatory substances from the egg cytoplasm bind to receptor sequences adjacent to the structural genes, and thus induce their initial derepression. When the parentally derived receptor sequences are identical, the two alleles at the respective structural gene locus become activated synchronously. But when the base sequence of the paternally derived receptor site is different from the maternally transmitted one, for example when the two haploid genomes stem from different species, the recognition of the paternally derived receptor sequence by the regulatory substance of the egg might become difficult or even impossible. This would lead to either transitory or permanent exclusive expression of the maternally transmitted structural gene. Using the first appearance of embryonically specified α -glycerophosphate dehydrogenase (α -GPDH) isozymes as markers during the ontogeny of interspecific hybrid trout, we have now observed preferential activation of the paternally derived alleles. This seems to contradict the model of gene activation outlined here.

Brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) are easily hybridised to produce viable but sterile

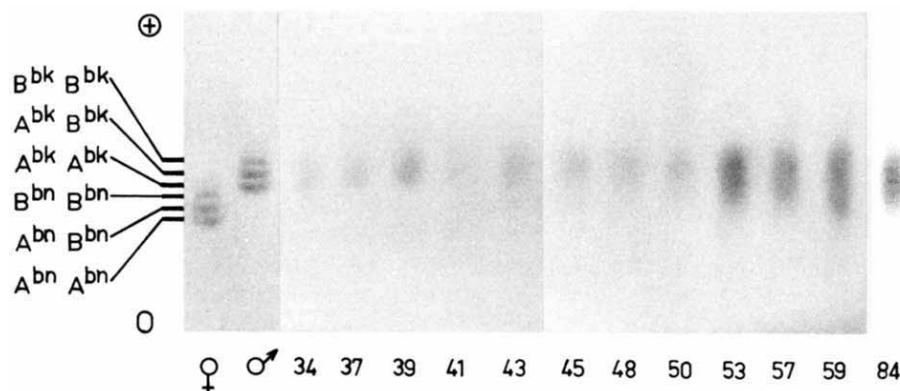


Fig. 1 Starch gel electrophoresis of α -GPDH isozymes from parental skeletal muscle tissue and single embryos at different developmental stages (age after fertilisation in days). Skeletal muscle (1:1 w/v) and embryos (about 1:0.25 w/v) were homogenised in 0.01 M phosphate buffer, pH 7.5, containing 10^{-4} M MgCl_2 . The 20,000g (skeletal muscle) and 10,500g (embryos) supernatants were subjected to electrophoresis. Gels were made of 14% starch in 0.008 M Tris-0.003 M citrate buffer, pH 8.0; bridge: 0.223 M Tris-0.068 M citrate buffer, pH 8.0; run for 5 h at 12 V cm^{-1} . Gel slices were stained as described before⁹.

hybrids. The time sequence of embryonic development in the offspring of intraspecific and interspecific matings is identical⁸. Both species have two ubiquitously expressed gene loci coding for a α -GPDH (EC 1.1.1.8), an enzyme of apparently dimeric structure^{8,9}. The wild-type isozyme patterns in the two species consist of electrophoretically distinct triplets, of which the subunit designations are given in Fig. 1.

No α -GPDH staining activity was observed in unfertilised brown trout eggs. In the offspring of a mating between a female brown trout (phenotype $A^{bn}B^{bn}$) and a male brook trout (phenotype $A^{bk}B^{bk}$) staining activity was observed for the first time on day 34 after fertilisation (Witschi stage 20). The stain clearly was located in the position of the paternal type of isozymes, and the pattern remained unchanged until day 52. On day 53 (Witschi stage 23) enzyme activity in the position of the maternal species was visible for the first time. On day 84 (shortly before the resorption of the yolk sac) all of a sample of ten embryos showed the expected $A^{bn}B^{bn}$ $A^{bk}B^{bk}$ phenotype, exhibiting an electrophoretic pattern of seven bands (Fig. 1). Our study was extended also to the offspring of the reciprocal and the intraspecific matings. In these cases the maternally and paternally derived α -GPDH genes seemed to become activated simultaneously. (These findings will be reported elsewhere in detail.)

The preferential activation of paternally derived α -GPDH alleles seems incompatible with the model of gene activation in embryonic development outlined above. If, however, one assumes that a genetic polymorphism exists at a common receptor site adjacent to both of the two α -GPDH structural loci (preliminary results indicate close linkage of the two loci⁸) our finding becomes reconcilable with the model. The brown trout mother should have passed on to all of her offspring a variant receptor sequence, characterised by a lowered affinity to the regulatory substance compared with the paternally transmitted receptor sequence. Only when the cellular concentration of the regulatory substance has reached a threshold, possibly also by synthetic activity of the embryonic genome, can the maternally derived α -GPDH loci be switched on.

It is conceivable that mutational events could alter receptor sequences so that they will never be recognised by the regulatory substances. In fact, permanent repression of the maternal allele was observed in interspecific *Drosophila*¹⁰ and sunfish^{11,12} hybrids. Whitt *et al.*¹² suggested that permanent maternal allele inhibition is due to a repressor molecule produced by the paternal genome which, when associated with the paternal DNA, can react appropriately to specific gene-activating stimuli, but when associated with the maternal DNA, with a different nucleo-

tide sequence, cannot respond. This model, based on observations with adult animals is obviously difficult to apply to early development, whereas genetic variability at receptor sequences might explain either set of findings.

If receptor sequences can occur in different allelic forms, preferential activation of paternally inherited genes should also occur in the offspring of intraspecific matings. We are testing this hypothesis. We thank Fischzuchtmeister H. Scheiffelen, Alpirsbach, Black Forest, for breeding the hybrids.

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Increased frequency of heterozygotes for α_1 antitrypsin variants in individuals with either sex chromosome mosaicism or trisomy 21

THERE have been two reports^{1,2} of an association of alpha-1 antitrypsin (α_1 AT) variants and sex chromosome mosaicism. The data suggest that decreased α_1 AT activity, as found in individuals heterozygous or homozygous for α_1 AT variants, gives rise to abnormal chromosome segregation during mitosis. We report here further data which support those previous studies and suggest that decreased α_1 AT activity is also an aetiological factor in trisomy 21.

α_1 AT is produced by the liver and found in blood, semen, cervical mucus and other body fluids. It is a readily detectable, polymorphic, codominantly inherited inhibitor of cellular proteases. The structural gene for α_1 AT is not on chromosome 21; however, its exact location is not known at present³.

Using acid starch gel electrophoresis⁴ and immunofixation electrophoresis at pH 8.6 (ref. 5), we found that three of

nineteen individuals with mosaic sex chromosome abnormalities had a heterozygous α_1 AT phenotype (Table 1). Of the three, two had the M_{LAMB} variant of α_1 AT. M_{LAMB} is a newly discovered variant with approximately 20% less activity than the usual M form of α_1 AT and, in one survey, was found in less than 1% of individuals typed³. Given an average frequency of heterozygotes for all α_1 AT variants of approximately 10% (ref. 6), the observed number of α_1 AT heterozygotes (12) in the three studies of individuals with mosaic sex chromosome abnormalities ($N = 47$) is significantly greater than expected: $\chi^2 = 12.6$, $P = <0.001$. (For this and subsequent calculations we have assumed the expected population frequency of heterozygotes for α_1 AT to be exactly 10%.)

Table 1 α_1 AT phenotypes in patients with sex chromosome mosaicism

	α_1 AT phenotype		
	MM	MM_{LAMB}	MZ
45,X/46,XX	1	1	
45,X/46,X,i(Xq)	4		1
45,X/46,X,i(Xq)/47,X,i(Xq),i(Xq)	1		
45,X/46,X,r(X)	2	1	
45,X/46,XY	4		
45,X/46,X,t(22qYp)*	1		
46,XY/47,XXY	1		
46,XY/47,YYY	2		

*Short arm of Y translocated to long arm of 22.

We have also performed α_1 AT phenotyping on 60 institutionalised patients with karyotype proven trisomy 21. Since advanced maternal age markedly affects the incidence of Down's syndrome, we collected samples from two groups of patients with trisomy 21. Group 1 consisted of 29 patients whose mothers were under 30 yr old at the time of the patient's birth (mean maternal age $\pm$ s.e. = 25.0 ± 0.51 yr). Group 2 consisted of 31 patients whose mothers were older than 35 yr at the time of the patient's birth (mean maternal age $\pm$ s.e. = 40.0 ± 0.38 yr). The mean ages of the patients in the two groups (29.7 ± 1.9 and 26.4 ± 1.8 yr, respectively) were not significantly different. α_1 AT phenotyping was performed blind with respect to maternal age, and the age and sex of the patient. We found two α_1 AT heterozygotes in group 1 and 14 in group 2 (Table 2). The frequency of α_1 AT heterozygotes in the total of both groups is significantly greater than that of the general population ($P = <0.0001$). But the two groups are significantly different from each other ($P = <0.005$). Group 1 alone, compared with the general population, had no greater frequency of α_1 AT heterozygotes; group 2 had a markedly greater frequency of α_1 AT heterozygotes ($P = <0.0001$). Thus the significant association of trisomy 21 with variant α_1 AT alleles seems to be confined to individuals born to mothers over 35.

These observations, if supported by more extensive studies, raise additional questions. How do advanced parental age and diminished α_1 AT activity (associated with heterozygous variants of α_1 AT) interact to produce chromosomally abnormal offspring? Does diminished α_1 AT activity predispose to other

types of chromosome aberrations? What role, if any, does α_1 AT have in normal meiosis and mitosis? Answers to these questions will provide important insights into mechanisms which control chromosome segregation. One important practical benefit may be the identification by α_1 AT phenotyping of individuals with a significantly increased risk of producing children with chromosome abnormalities.

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Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocyte

In starfish, the first maturation division of oocytes within an ovary is arrested at a late stage of prophase. It is resumed after stimulation by 1-methyladenine (1-MA) produced in the follicle cells under the influence of the gonad-stimulating hormonal peptide, released from the nervous tissues¹⁻³. 1-MA induces breakdown of the germinal vesicle and maturation of oocytes isolated in seawater². When it causes maturation, 1-MA first acts on the oocyte surface^{4,5} to bring about cytoplasmic maturation, which is manifest in the acquisition of fertilisability⁶. The change in the cortical region of the oocyte under the influence of 1-MA is believed to produce a cytoplasmic factor which induces germinal vesicle breakdown (GVBD), leading to the completion of oocyte maturation. We now report micro-injection experiments that provide evidence of such a cytoplasmic factor in the starfish *Asterina pectinifera*.

An earlier experiment had shown that 1-MA injected directly into an immature oocyte failed to induce germinal vesicle breakdown even in a concentration higher than that which is effective when applied externally⁴. We found, however, that cytoplasm from an oocyte which is maturing after treatment with 1-MA can induce GVBD when injected into an immature oocyte by Hiramoto's method⁷ (Fig. 1). The frequency with which GVBD was induced in recipient oocytes was proportional to the volume of cytoplasm injected. Almost 100% breakdown was observed when the volume injected was 220 pl (about 1/15-1/20 of the oocyte volume). Germinal vesicle was broken down 20-40 min after microinjection, and so was follicular envelope, but GVBD was never observed when the cytoplasm of immature oocytes was injected as a control.

Cytological examination of oocytes which underwent

Table 2 α_1 AT phenotypes in patients with trisomy 21

Maternal age at the time of the patient's birth	α_1 AT phenotype	No. of patients	Frequency of heterozygotes
Group 1 less than 30 yr old	MS	1	0.069
	MM_{LAMB}	1	
	MM	27	
	Total	29	
Group 2 more than 35 yr old	MS	7	0.452
	$M_{LAMB}S$	1	
	MZ	2	
	$M_{LAMB}Z$	1	
	MM_{LAMB}	3	
	MM	17	
	Total	31	

GVBD after microinjection of cytoplasm from 1-MA-treated oocytes showed that meiosis was completed normally. Recipient oocytes were fixed with ethanol-acetic acid (1:1) and stained with aceto-orcein. Each oocyte was crushed under a cover glass and the chromosomes were observed microscopically. Chromosome condensation was observed 35 min, metaphase about 45 min and anaphase about 55 min after injection. The first and second polar bodies were discharged 70 min and 100 min after injection, respectively. The egg pronucleus was observed 120 min after injection. The time course of meiosis of injected oocytes was the same as that of oocytes treated with 1-MA in 80% artificial seawater. Thus the maturing oocyte produces a cytoplasmic factor which induces GVBD, leading to the completion of meiotic maturation. Furthermore, on insemination, oocytes induced to mature by injection of maturing cytoplasm formed somewhat incomplete fertilisation membranes. They then cleaved and developed normally.

Next we investigated the changes in the maturation-inducing capacity of the cytoplasm of 1-MA-treated oocytes as they matured. Cytoplasm (about 220 pl) of 1-MA-treated oocytes, taken at 20-min intervals after treatment, was injected into immature oocytes with germinal vesicles. When oocytes were taken from the same ovary and treated with 1-MA in the same way as donor oocytes, the germinal vesicle broke down after 15–30 min, the first polar body was discharged after 60–70 min and the second polar body was discharged after 110–120 min. Injected cytoplasm was most effective in inducing GVBD 20–40 min after administration of 1-MA, and then its efficacy declined (Fig. 2). After 80 min, the cytoplasm had almost lost the capacity to induce germinal vesicle breakdown when injected into immature oocytes, although recipient oocytes matured normally when subsequently transferred to seawater containing 10^{-6} M 1-MA. Furthermore, maturation-inducing capacity was found in the cytoplasm of some oocytes 13 min after administration of 1-MA, when germinal vesicles remained intact. The activity thus does not seem to be the result of GVBD.

Cytoplasm of an oocyte undergoing maturation as a result of microinjection of maturing cytoplasm was injected successively into immature oocytes. In three replicates about 220 pl of cytoplasm taken just after GVBD, was injected into an oocyte with intact germinal vesicle. Since this

Fig. 1 GVBD induced by microinjection of cytoplasm taken from 1-MA-treated oocytes of *A. pectinifera*. Donor oocytes were treated with artificial seawater (ASW) containing 10^{-5} M 1-MA for 10 min and washed with ASW. Cytoplasm was removed from donor oocytes 20–25 min after the commencement of treatment with 1-MA and injected into immature oocytes with intact germinal vesicle, which were then placed in 80% ASW. This concentration was more suitable than normal ASW for injecting a large quantity of cytoplasm. ●, Cytoplasm from 1-MA-treated oocyte; ○, cytoplasm from intact immature oocyte. Number of injected oocytes is shown in parentheses.

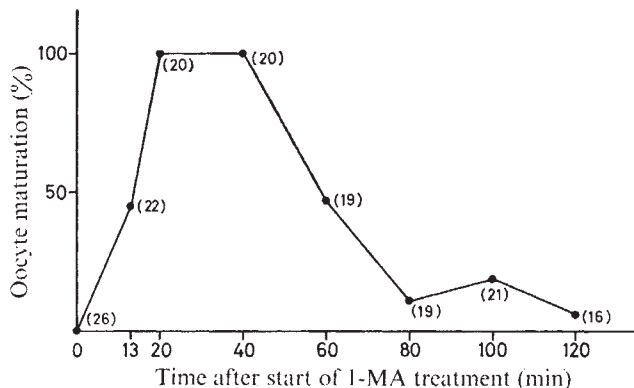
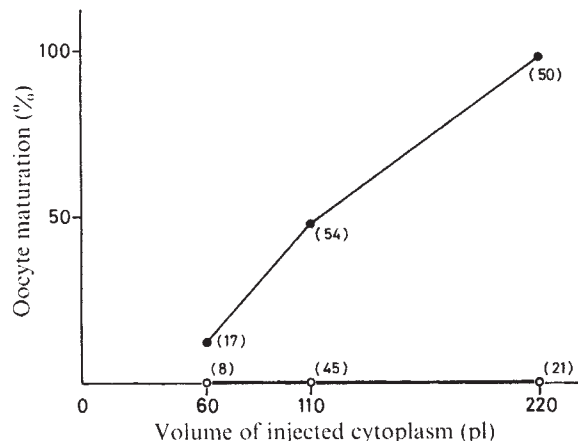


Fig. 2 Changes in the maturation-inducing capacity of the cytoplasm of 1-MA-treated oocytes during maturation in *A. pectinifera*. Donor oocytes were treated with ASW containing 10^{-5} M 1-MA for 10 min and washed with ASW. Number of injected oocytes is shown in parentheses.

volume of injected cytoplasm, 1/4 of which barely induced maturation (Fig. 1), was about 1/15 of the oocyte volume, the cytoplasm of the original oocyte which matured in response to 10^{-5} M 1-MA was diluted 15^4 times after the fourth injection. In spite of such extensive dilution, the rates of maturation of the recipient oocyte were, respectively, 100% (18 of 18), 88% (15 of 17), 93% (14 of 15) and 92% (12 of 13) in the first, second, third and fourth injections. Thus the cytoplasmic factor able to induce maturation was amplified in recipient oocytes during maturation.

These results are interesting when compared with data for oocyte maturation in frogs^{8,9}. In *Rana pipiens*, pituitary gonadotropins seem to act on the follicular tissue to produce progesterone or a related substance which brings about oocyte maturation. Injection of progesterone into the oocyte fails to induce maturation, whereas external application to oocytes free of follicle cells is effective. But the cytoplasm of progesterone-treated frog oocytes can also induce maturation when injected into immature oocytes. Therefore, progesterone is considered to act externally on the oocyte surface to produce a cytoplasmic factor called "maturation-promoting factor", which causes the oocytes of frogs to mature. In the starfish, gonad-stimulating hormonal peptide is secreted from the nervous system to produce 1-MA in the follicle cells. 1-MA acts on the oocyte surface to produce and release a new factor in the cytoplasm which is the true inducer of germinal vesicle breakdown and meiotic maturation. Further work should be directed towards the characterisation of this factor.

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Association of lipid oxidation with seed ageing and death

SEEDS usually exhibit their maximum germination potential soon after harvest, and as storage time increases, they lose vigour and eventually die. The rate of physiological ageing generally increases with increased moisture content and temperature¹. In addition, certain xerophytic storage fungi (primarily *Aspergillus* spp.) can attack seeds in storage when seed moisture content is in equilibrium with relative humidities greater than 65% (ref. 1), and cause changes that mimic physiological ageing. Physiological changes accompanying physiological ageing²⁻³ and attack by storage fungi⁴ have been catalogued, but the basic biochemical events leading to these changes are not known. Harrington¹ and Pammenter *et al.*⁶, however, have suggested that oxidation of unsaturated lipids may lead to free-radical formation. Free radicals may then damage cellular membranes and react destructively with macromolecules. Similar suggestions have been presented to explain ageing in several other systems⁷, and the results of experiments reported here indicate that free-radical formation may be important in seed deterioration. Theories of mechanisms of lipid oxidation indicate that fatty acids with two or more unsaturated bonds should be more labile and prone to form free radicals than more highly saturated acids^{8,9}. Thus, if fatty acid oxidation and free-radical formation are occurring, the highly unsaturated acids should decrease as seeds deteriorate, whereas the quantity of saturated fats should remain constant in the absence of beta oxidation.

To determine whether these predicted changes occurred as a result of ageing and/or of infection by storage fungi, we used pea (*Pisum sativum* L. 'Alaska') seeds rendered microorganism-free or inoculated with *Aspergillus ruber* (Konig, Spieckermann, and Bremer) Thom and Church prepared as described elsewhere⁵. They were stored at 30 °C and 92% relative humidity. Periodically, seeds of different ages were removed from storage, and the percentage germination, the vigour, and the quantities of the major fatty acids in whole seeds and embryonic axes were determined. Seeds inoculated with *A. ruber* were sampled only at 8 weeks, since visible infection (sporulation from the hila) does not commence until at least 6 weeks after inoculation. Only seeds with visible sporulation were used in these experiments. The percentage germination was determined by placing 30 seeds between sterile, wet paper towels, and counting the number of seeds germinating normally after 5 d incubation at 25 °C. A vigour rating was obtained by placing other seeds in the same conditions as for germination testing, and measuring the increase in fresh weight of the shoot-root axes after 3 d growth. This method measures the speed of germination and is correlated with several biochemical tests for vigour⁵.

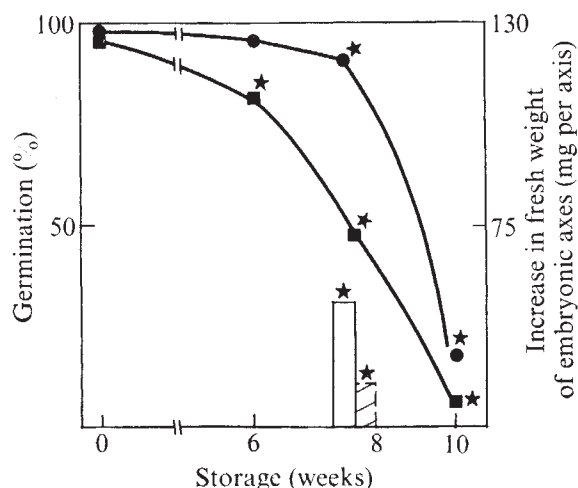
To determine the quantities of fatty acids, seeds were first lyophilised, and then ground to a fine powder in a Waring Blender. Hydrolysis, methylation of fatty acids and extraction of the resulting esters was accomplished by a modification of the method of van Wijngaarden¹⁰. An aliquot (400 mg) of each subsample of ground pea seeds was placed in a 50-ml round bottomed flask, and 3 ml of 0.66 N methanolic KOH and 1 ml of heptadecanoic acid in methanol (1 mg ml⁻¹ as an internal standard) were added to the flask. The flask was then connected to a condenser, the mixture was brought to a slow boil for 5 min, 4 ml of BF₃ in methanol (14% w/v) was added through the condenser, and the mixture was boiled for a further 2 min. Heptane was then added (1 ml), again through the condenser, the mixture boiled for an additional minute, and the condenser and flask then cooled rapidly in an ice bath. Sufficient saturated NaCl solution was added to the cooled mixture to bring it to the neck of the flask, and an aliquot

of the heptane layer transferred to a vial containing a small amount of anhydrous Na₂SO₄. This heptane solution contained the fatty acid esters and was injected directly into the gas chromatograph. Separation was accomplished on a column (32 mm outside diameter and 1.8 m long) of Chromosorb WHP 100-120 mesh coated with 5% diethylene glycol succinate maintained at 150 °C. The carrier gas was N₂ at 10 ml min⁻¹. Detection was by a flame ionisation detector and quantitation was by integration of the area of each peak, corrected for the sensitivity of the detector relative to a heptadecanoic acid standard (determined earlier using known quantities of each of the fatty acid esters). The identities of the acid esters were verified by chromatography on 1% SE-30 (methyl), a non-polar gum rubber, using the same conditions as for the diethylene glycol succinate column. All experiments were conducted in triplicate.

The percentage germination of microorganism-free pea seeds decreased gradually from 0 to 8 weeks of storage, and then decreased markedly between 8 and 10 weeks (Fig. 1). Vigour, however, had decreased noticeably by 6 weeks of storage and declined more rapidly than germination during 8 weeks of storage. Between 8 and 10 weeks, vigour, like germination, decreased rapidly. The rapid decline of vigour compared with viability (germination) as a consequence of ageing has long been noted¹², but its cause is unknown. Seeds infected with *A. ruber* showed reduced vigour and germination compared with similar seeds stored in aseptic conditions.

The principal fatty acids of pea seeds were identified as palmitic, stearic, oleic, linoleic and linolenic acids. No treatment changed the concentrations of the saturated or monoenoic acids (palmitic, stearic, and oleic) in whole seeds or embryonic axes. The concentrations of these acids in whole seeds were 2.0, 0.50, and 5.1 mg per g dry weight, and those in axes were 1.8, 0.25, and 5.8 mg per g dry weight, respectively. The concentration of both the di- and trienoic acids (linoleic and linolenic), however, were affected by both storage and infection (Fig. 2). In whole seeds, the concentration of linoleic acid declined gradually with increased storage, and there was no additional decrease after infection. There was no significant change in linoleic acid concentration in the axes, larger changes were observed with linolenic acid: in whole seeds, only about 50% of linolenic acid remained after storage for 10 weeks with the most dramatic decrease of this acid occurring between

Fig. 1 Percentage germination of microorganism-free (●) and *Aspergillus ruber*-infected seeds (open column), and the increase in fresh weight of embryonic axes from microorganism-free (■) and *A. ruber*-infected seeds (hatched column) after 3 d germination. Starred points (★) are significantly different ($K = 100$) from values obtained from unaged seeds according to the test of Waller and Duncan¹⁴.



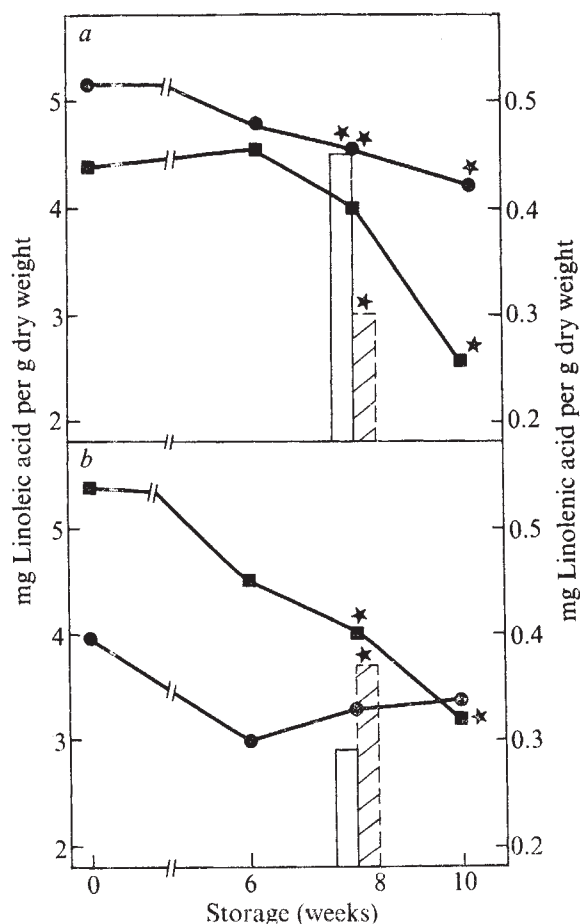


Fig. 2 Changes in the concentration of linoleic and linolenic acids in whole pea seeds (a) and in embryonic axes (b) after storage for various times. The concentration of linoleic acid in microorganism-free (●) and in *Aspergillus ruber*-infected (open column), and the concentrations of linolenic acid in microorganism-free (■) and *A. ruber*-infected (hatched column) seeds are shown. Starred points (★) are significantly different ($K = 100$) from the levels obtained for unaged seeds according to the test of Waller and Duncan¹⁴.

weeks 8 and 10. In embryonic axes, there was a rapid decrease in the quantity of linolenic acid with ageing, but no additional decrease as a result of infection by *A. ruber*.

These results suggest strongly that beta oxidation or other utilisation of fatty acids does not occur in stored seeds, since the saturated and monoenoic acids did not change in concentration. The rapid decrease in linolenic acid with ageing in axes roughly paralleled the loss of vigour of the seeds and suggests that free-radical formation may be a factor in loss of seed vigour and, subsequently, seed death. Changes occurring in the axes are probably of more consequence to the overall health of the seed than those occurring in the whole seeds (cotyledons, axes and seed coat), since only the axes are capable of growth. They should also be more sensitive to oxidation processes since they are rather small structures, comprising ~1% of the total seed weight, attached to the outer surface of the embryo. Toxic materials formed in the cotyledons as a consequence of oxidation could, however, be transported to the axes and cause damage.

Changes in linoleic acid were less pronounced than changes in linolenic acid; this was expected since linoleic acid is less reactive than linolenic acid⁹, and since it is present in an approximately tenfold higher concentration than linolenic, relatively small changes (less than ~0.5 mg g⁻¹) are difficult to detect.

A. ruber infection caused a statistically significant reduction in the quantity of linolenic acid in whole seeds, but not in axes (Fig. 2). Any explanation of this phenomenon must

consider that *A. ruber* damages pea embryos by way of a diffusible substance, since the fungus is restricted to the seed coats and rarely invades living embryos¹³. The *A. ruber* toxin could somehow initiate oxidation without a pronounced effect on the subsequent oxidation rate. Thus, in axes where oxidation due to ageing occurred before infection was heavy, lipid oxidation would not be strongly affected by *A. ruber* infection, whereas in whole seeds (primarily cotyledons), the processes of initiation could still be occurring. More information on the mechanisms of oxidation as a consequence of ageing and infection is needed before any conclusions can be drawn on the relative effects of ageing or infection on the oxidation of fatty acids.

The correlations shown here, particularly between the disappearance of linolenic acid, loss of seed vigour and seed death, thus indicate that free radical formation may well be an important factor in seed deterioration.

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Enrichment of antigen-specific helper and suppressor T cells

LYMPHOCYTES are known to differ on the basis of their antigen specificity, as postulated by Burnet¹, and experimentally confirmed by a variety of techniques²⁻⁵. Although there are many problems involved in purifying cells reactive to a given antigen there have been successful reports. These include the purification of rosette-forming cells⁶, the separation of cells binding fluoresceinated antigen on a cell sorter⁷, and the recovery of cells which have bound to an antigen matrix⁸ or a cell surface⁸⁻¹⁰. The use of gelatin^{8,11} substrates has proved to be successful in the purification of B lymphocytes, with enrichment of antigen-specific cells estimated in the order of 100-fold^{11,12}. Because antigen-binding T cells bind better at 37 °C than at 4 °C (refs 13 and 15), collagen gels were used in this study, as gelatin melts at 37 °C. A procedure for the enrichment of antigen-specific T cells should provide an essential step in the approach to many problems of understanding T cells and their function, such as the nature of T-cell receptors on subpopulations of T cells, the nature of mediators of T-cell function (especially antigen-specific mediators) and the mechanisms and control of T-cell activation. Here we describe a method for the selective enrichment of T cells reactive to protein or synthetic polypeptide antigens, and demonstrate the selectivity and specificity of the procedure by cross absorption experiments. The general applicability of the procedure was verified by using T cells immunised

in vivo or *in vitro*, and by demonstrating 100-fold enrichment with different subpopulations of T cells, namely carrier-specific helper and carrier-specific suppressor cells involved in the *in vitro* antibody response.

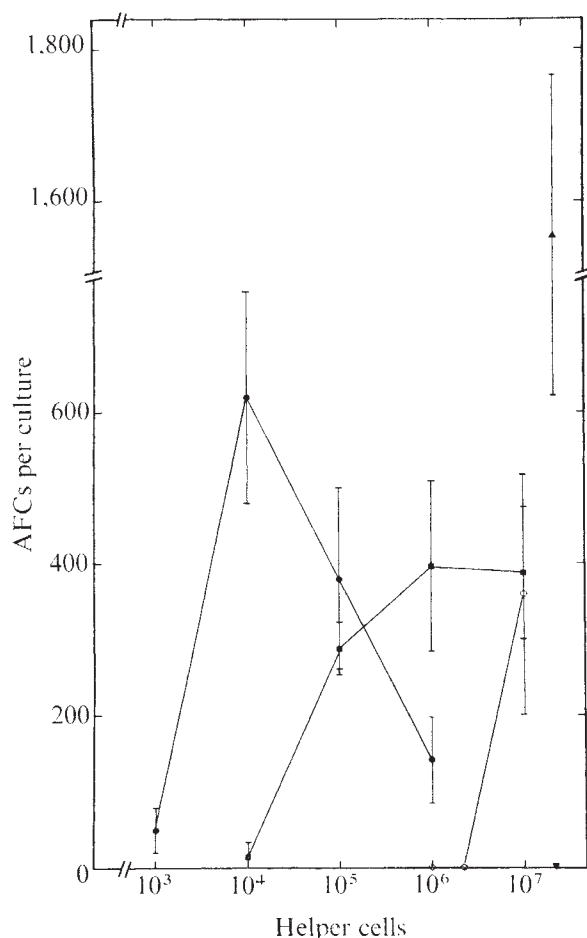


Fig. 1 Enrichment of helper cells from mice primed *in vivo*. ●, Adherent cells; ■, unfractionated cells; ○, non-adherent cells. Spleen T cells from mice primed 3 times with TNP-KLH adsorbed on bentonite³⁷ were passed through nylon wool²⁸. The passed cells contained 4% cells stainable with fluoresceinated anti-Ig (B cells) and 95% cells stainable with anti θ . These cells were plated on keyhole limpet haemocyanin (KLH) derivatised collagen gels. Cell suspensions (20×10^6 – 35×10^6 cells per 2 ml) in HEPES (15 mM) buffered Eagle's (HEM) were incubated on a rocker for 60 min at 37 °C. Non-adherent cells were removed by aspiration followed by 2 washes with HEM. Adherent cells were recovered from the gels by digestion with collagenase (Worthington Biochemicals Co.), 180 μ g per plate in 2.0 ml HEM for 90 min at 37 °C, followed by centrifugation and resuspension in a small volume of HEM containing 10% foetal calf serum. The specific binding of the populations used was 1–2% of the cells applied, and nonspecific binding, to collagen itself, or derivatised collagen (with another protein) was 0.5%. The helper capacity of the 2 fractions was compared with that of the unfractionated T cell *in vitro*, using unprimed CBA spleen (sometimes anti-T treated spleen) as a source of B cells and 0.1 μ g ml⁻¹ trinitrophenylated KLH (TNP-KLH) as antigen. Cultures were performed in Marbrook-Diener flasks as described in detail elsewhere^{25,29}. Antibody-forming cells to DNP or TNP were assayed after 4 d in culture^{25,29}. Gels used in this study were made from acid-soluble rat tail tendon collagen, 0.1% in 0.1 M acetic acid, layered in Petri dishes (usually Sterilin 50 mm), and the gels formed by exposure to ammonia. Binding of antigen to the gels was performed following periodate oxidation of the gels with 0.1% sodium periodate (BDH) for 1 h at room temperature in the dark. The gels were washed with phosphate-buffered saline (PBS) and the protein solution (usually 0.1–1.0 mg ml⁻¹) was added and left overnight. The gels were then washed with PBS and HEM, and usually used immediately. Occasionally they were stored in the cold for up to 4–5 d. Arithmetic mean $\pm$ s.e.m. of 3 cultures per group (for all groups). Analogous results were obtained in 7 experiments of this type with KLH and 5 with chicken gamma globulin.

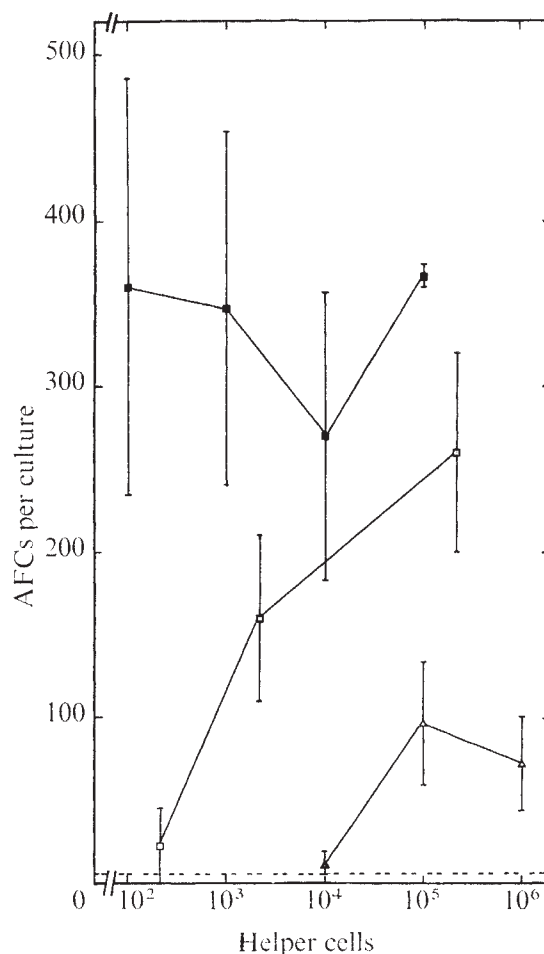


Fig. 2 Enrichment of *in vitro* primed helper cells to KLH. ■, Adherent cells; □, unfractionated cells; △, non-adherent cells. T-helper cells were induced by culture for 4 d in Marbrook flasks with low concentrations of antigen¹⁶. Surviving cells were plated on KLH gels and the adherent and non-adherent fractions were collected and compared for their helper capacity *in vitro*, as described in the legend to Fig. 1. As few as 100 adherent cells sufficed for an optimal helper response, compared with 10⁵ of the unfractionated cells. 10⁶ non-adherent cells were less efficient than 10² adherent, verifying that the technique both enriches and depletes for antigen-specific cells. Note that the depletion in this (and all other) experiments is not complete. Three experiments of this type have been performed with analogous results.

Nylon wool-purified T cells from mice primed to keyhole limpet haemocyanin (KLH) were incubated with KLH derivatised collagen (1 mg per 50-mm dish was found to be optimal) for 60 min on a rocker at 37 °C. The non-adherent cells were removed and the gels rinsed and digested with collagenase. In all experiments the viability of the adherent cells was about 90%.

The helper capacity of three fractions were compared: (1) the nylon-purified T cells, (2) the cells adherent to the KLH and (3) the non-adherent cells. Titrations were performed *in vitro*, comparing the numbers of *in vivo* primed T cells needed to generate anti-dinitrophenyl (anti-DNP) antibody responses, using normal spleen as a source of antibody-forming cell (AFC) precursors and triphenylated KLH (TNP-KLH) as antigen. As shown in Fig. 1, the adherent cells were about 30 times more efficient helper cells than the unfractionated T cells. The T cells which did not adhere to the KLH gels were depleted of helper cells by about 100-fold.

Analogous experiments were also performed with helper cells obtained from tissue culture¹⁶. These helper cells, perhaps being more recently in contact with antigen, are effective in smaller numbers (optimum in range 10⁴ to 5 $\times$ 10⁵) than helper cells from primed mice. Such cells

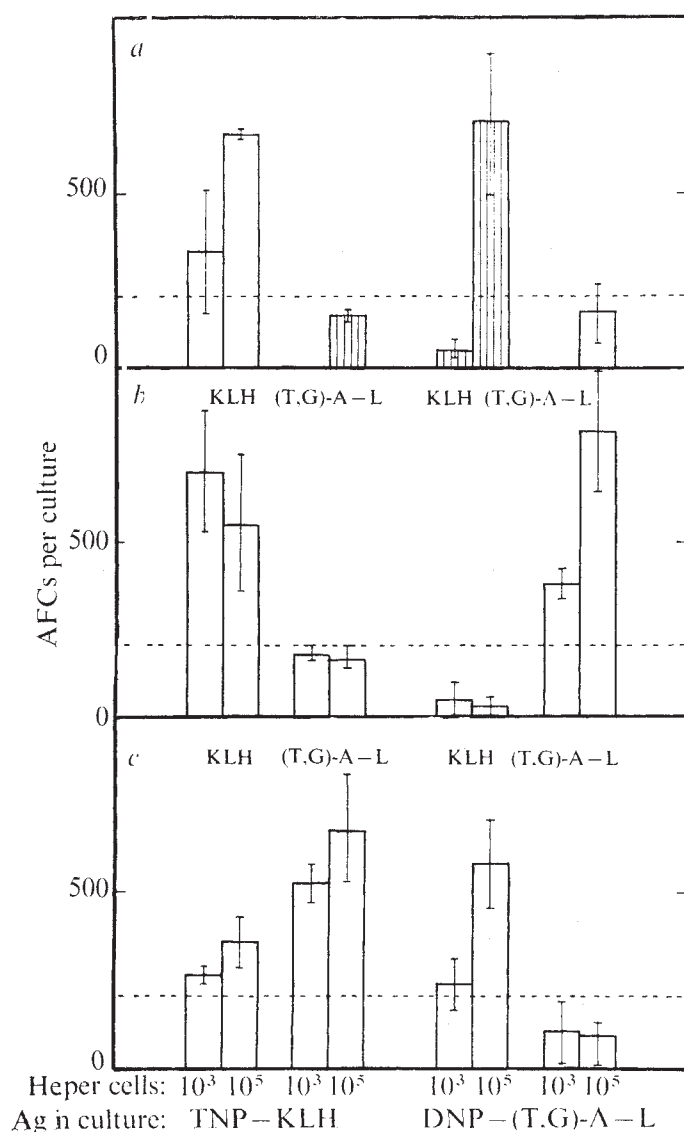


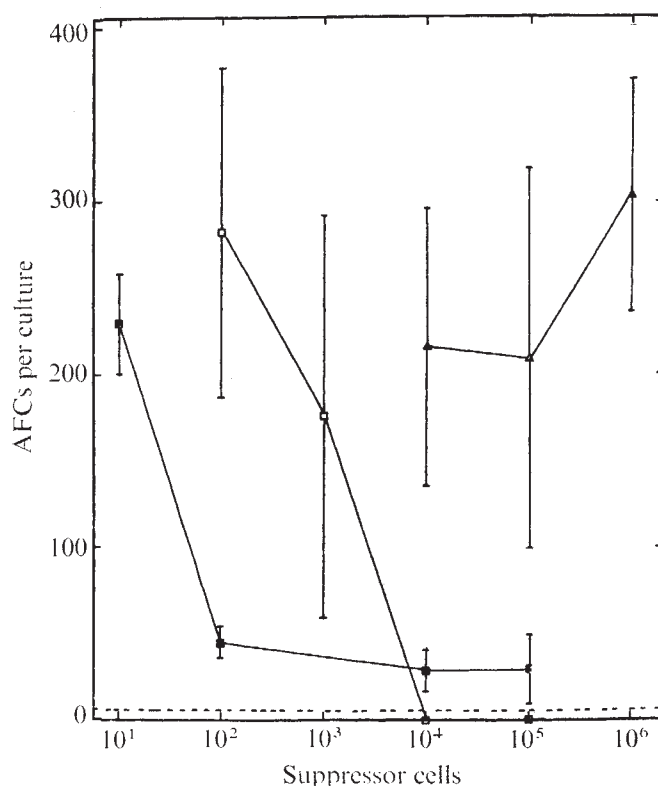
Fig. 3 Specificity of the enrichment of *in vitro* induced helper cells. Spleen cells were cultured with either KLH or (T,G)-A-L to induce helper cells. To determine the specificity of binding of helper cells to antigen-coated gels, KLH and (T,G)-A-L specific helper cells were mixed in equal numbers and applied to either KLH or (T,G)-A-L gels. The adherent and non-adherent cells were tested for their helper capacity for both (T,G)-A-L and KLH by culture with spleen cells containing $1.0 \mu\text{g ml}^{-1}$ DNP-(T,G)-A-L and $0.1 \mu\text{g ml}^{-1}$ TNP-KLH respectively. Cells adherent (b) to KLH gels provided excellent help when challenged with TNP-KLH (for example, 700 AFC with 10^3 cells) but did not help when challenged with DNP-(T,G)-A-L (180 with 10^3 cells) thus verifying the specificity of the absorption. In a reciprocal way, cells non-adherent (c) to KLH gels provided better help when stimulated with DNP-(T,G)-A-L than with TNP-KLH. a, Unfractionated cells, plated on KLH or (T,G)-A-L gels, and tested as shown. Two cell doses were used (10^3 and 10^5). Two experiments of this type have yielded analogous results. Background (no helper cells added) is indicated by dotted lines.

were incubated with KLH collagen, as described (see legend to Fig. 1) and divided into adherent and non-adherent populations. The adherent cells were highly efficient helper cells, with 100 cells sufficing for an optimal response (Fig. 2). This represents an enrichment factor of $\sim 10^3$. Repeat experiments have yielded analogous results, with the enrichment varying from 100- to 1,000-fold. The depletion of helper cells is hard to assess precisely as it is not possible to add very large numbers of cultured helper cells to the test cultures, because of the suppressive effects of these cells if added in excess of 10^6 . In Fig. 2 the depletion is 100-fold. In other experiments it has ranged from 10- to 100-fold.

These experiments demonstrated the capacity of the technique to enrich for KLH reactive cells, or in other experiments, chicken gamma globulin or cells reactive to (T,G)-A-L (a random copolymer of L-tyrosine, glutamic acid, coupled to DL-alanine side chains, attached to poly L-lysine backbone) have been purified. Since there was appreciable nonspecific binding ($\sim 0.5\%$) it was of importance to establish that the cells binding nonspecifically do not belong to a given subpopulation of T cells. It could be argued, for example, that helper cells (of any antigenic specificity) are preferentially retained on collagen. To exclude this possibility, and to demonstrate further the specificity of cell selection, helper cells immunised to KLH and (T,G)-A-L *in vitro* were mixed, and the mixture plated on KLH or (T,G)-A-L gels. Adherent and non-adherent cells were recovered from each, and both fractions tested with both DNP-(T,G)-A-L (for (T,G)-A-L helper cells) and TNP-KLH, for KLH helper cells. The results (Fig. 3) show that there is no detectable cross contamination of enriched helper-cell populations. Since as many as 10^5 adherent cells to one antigen, KLH, did not stimulate responses to (T,G)-A-L whereas 10^3 were sufficient for optimal help to KLH, the degree of contamination was less than 1%. Results of this type indicate that the usual nonspecific binding ($\sim 0.5\%$) is not selective for helper cells in general, and that the enrichment for helper cells is through their antigen specificity.

Another major class of T cells involved in antibody responses are suppressor cells (reviewed in ref. 17). These may be specific or nonspecific. It was of interest to ascertain

Fig. 4 Enrichment of KLH-specific suppressor cells induced *in vitro*. Suppressor cells were induced by incubation of CBA mouse spleen cell with $100 \mu\text{g ml}^{-1}$ KLH for 4 d *in vitro*³⁰. These cells suppress the action of KLH helper cells *in vitro* in an antigen-specific manner, and are assayed by their capacity to suppress the cooperative response of (usually) 3×10^5 *in vitro* educated helper cells and 15×10^6 normal spleen cells stimulated with $0.1 \mu\text{g ml}^{-1}$ TNP-KLH. As shown 10^4 - 10^5 suppressors suffice to inhibit this response. After adherence to KLH gels the efficiency of these cells was augmented 100-fold; likewise the non-adherent cells were depleted of suppressor cells by 100-fold.



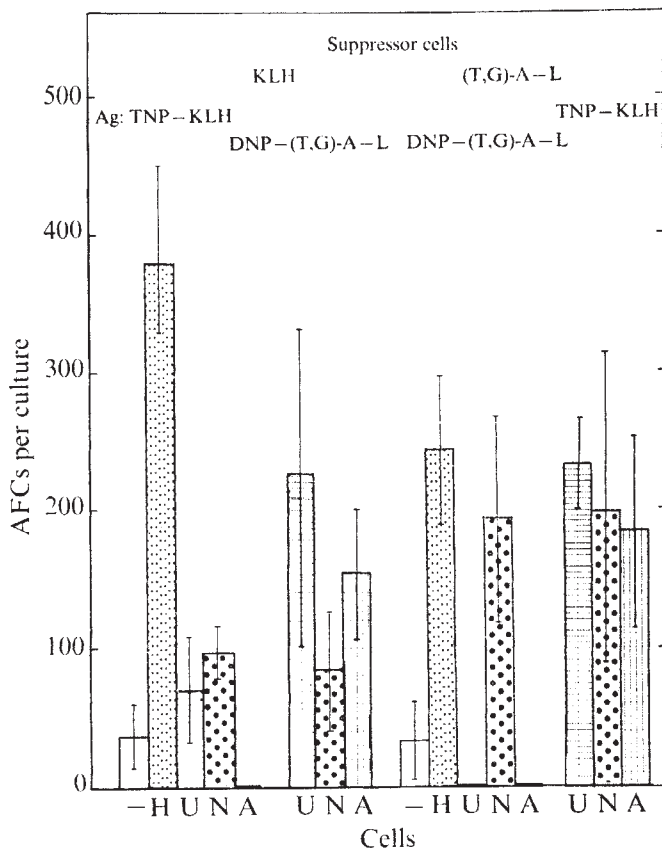


Fig. 5 Specificity of the enrichment of suppressor cells. Suppressor cells were induced *in vitro* to KLH and (T,G)-A-L. A mixture of suppressor cells were plated on KLH and (T,G)-A-L gels, and the adherent and non-adherent cells tested for their suppressor activity directed to KLH or (T,G)-A-L. As shown, KLH suppressors inhibited the response to TNP-KLH but not to DNP-(T,G)-A-L. Cells which adhered to KLH gels suppressed the response to KLH, but not to DNP-(T,G)-A-L, and vice versa. For simplicity, the results with 10^5 cells are shown. By dilution analysis the degree of specificity is >100 -fold. —, Background; H, helper cells, U, unfractionated, suppressor cells; N, non-adherent; A, adherent suppressor cells. Two experiments of this type have been performed with analogous results.

whether suppressor T cells could also be partly purified with antigen coated gels. Suppressor cells specific for KLH were obtained from cultures stimulated with a high concentration of KLH. These suppressor cells are assayed by their capacity to inhibit the response induced by KLH-specific helper cells. Figure 4 indicates that small numbers (10^3 – 10^4) of such suppressor cells suffice to abolish the response totally. After adherence and recovery from KLH gels, as few as 100 cells were sufficient to abolish the response, again yielding an enrichment factor of 100-fold. Repeat experiments yielded enrichments of 100–1,000-fold. The degree of depletion of suppressor cells, was, however much less satisfactory, probably because so few cells suffice for suppression. The results have varied, but a single passage on KLH gel resulted in 10–100-fold depletion, more usually nearer tenfold.

To test the specificity of the purification of suppressor cells, a mixture of KLH and (T,G)-A-L suppressor cells was purified on KLH or (T,G)-A-L gels, and the unfractionated, adherent and non-adherent (to both gels) cells were tested for suppression of the response to DNP-(T,G)-A-L or TNP-KLH. Figure 5 illustrates such an experiment, which indicates that only KLH suppressors bound to KLH, and (T,G)-A-L suppressors to (T,G)-A-L. By dilution analysis, the degree of contamination was less than 1% (full data not shown).

The results indicate that collagen is a useful substrate for

the partial purification of antigen-specific T cells. It was successful with T cells primed *in vivo* or *in vitro*, with two different functional subclasses of T cells, helper cells and suppressor cells (which have recently been shown¹⁸ to be phenotypically different).

The difficulty in demonstrating antigen-binding T cells^{13,15}, taken together with the increasing evidence that mouse cytotoxic T cells preferentially recognise H-2D or H-2K specificities (see for example ref. 19) and virus^{20,22} or hapten modification²³, thereof, and since T cells cannot respond to antigen in the absence of macrophages^{24,25}, it has been suggested that, unlike B cells, T cells may only respond to antigen-modified self components, particularly transplantation antigens. Because we have succeeded in purifying helper T cells on antigen-collagen gels and this binding is blocked (by $>90\%$) by free antigen (to be published) it is clear that T-helper cells, at least, do have receptors for the antigen (for example KLH) *per se*. Similar considerations apply to antigen-collagen purified suppressor cells, and the results (Figs 4 and 5) together with analogous experiments performed with suppressor cell populations, in the absence of helper cells (which were killed by anti-Lyl antiserum¹⁸) indicate that suppressor cells can recognise antigen, and eliminate the possibility that suppressor cells can only recognise idiotype determinants²⁶.

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Immunoglobulin-bearing cells in bone marrow of mice after prolonged treatment with anti-IgM antibodies

In the chicken, differentiation of immunoglobulin (Ig)-bearing cells occurs in the bursa Fabricius^{1,2}. Removal of the bursa at various times in the perinatal period³⁻⁵ and/or administration of anti- μ chain antibodies have led to the postulate that IgM-bearing cells give rise in a sequential manner to cells with IgG and IgA on their surface⁶. Thus, administration of anti- μ to chicken embryos results in the elimination of cells bearing Ig of all classes, and in a depression in the synthesis of both IgM and IgG⁷. Administration of anti- μ to neonatal mice has also been shown to lead to a decrease in the serum level of all Ig classes, as well as a reduction of all Ig-bearing cells in the spleen, suggesting that the maturation sequence of Ig-bearing cells in birds and in mammals may be similar⁸⁻¹⁰. Recent work by one of us (D.G.O.) has shown that in mice the bone marrow is a site of production and differentiation of Ig-bearing lymphocytes, suggesting that the bone marrow may be a mammalian equivalent of the bursa of Fabricius¹¹⁻¹³. The aim of the present investigation was to establish the distribution of lymphocytes bearing various Ig classes in the bone marrow of mice treated with anti-IgM, and compare it with that in normal bone marrow. The results indicate that anti-IgM injections prevent the development of lymphocytes bearing Ig of all classes in mouse bone marrow. In addition, most of the "null" small lymphocytes and many large lymphoid cells, hitherto found to be devoid of detectable surface Ig¹¹⁻¹⁴, are also eliminated.

The anti-IgM serum administered to mice was produced in rabbits by methods modified from those of Murgita *et al.*⁸ and Crowle *et al.*¹⁵. IgM was purified from the sera of BALB/c mice bearing myeloma 104E. Specific precipitates of the IgM with rabbit anti-IgM serum were injected into rabbits twice, 2 weeks apart, and were followed by a booster injection of 1 ml BALB/c serum. The animals were bled 5 d later. Serum, pooled from 64 rabbits, was twice precipitated with ammonium sulphate at 50% and 33% saturation respectively, absorbed with rat and mouse erythrocytes to remove natural red cell agglutinins, Millipore filtered, and stored in the frozen state. The final preparation had a protein concentration of 138 mg ml⁻¹; in gel diffusion in a dilution of 1:32 it reacted with a purified IgM preparation (Bionetics Laboratory Products), while against BALB/c serum it gave a single line of precipitation identified by immunoelectrophoresis as IgM.

The mice used in all the experiments were DBA/2 (Jackson Laboratories). One-day-old mice were injected intraperitoneally with 5 mg or 10 mg anti-IgM globulin, followed by maintenance injections of the same amount, three times weekly. A second group of experimental animals consisted of 9-week-old males, X irradiated (950 r.) and reconstituted with 2, 5 or 10 $\times 10^6$ syngeneic bone marrow cells. They received 75 mg or 200 mg anti-IgM globulin 5 d later, followed by maintenance injections of 20 mg antibody globulin 3 times weekly. Controls consisted of untreated mice, or X-irradiated mice, repopulated with bone marrow, and injected with physiological saline.

Autoradiography of antiglobulin-binding cells was performed as described previously¹⁴. Antibody used to detect IgM-bearing cells in the first group of mice was purified by chromatography on DEAE-Sephadex from the rabbit anti-IgM pool used to inject the mice. The antisera used for the second group of animals were monospecific anti- μ , anti- γ 1, anti- γ 2 and anti- α (Meloy Laboratories), precipitated with ammonium sulphate at 50% saturation. All

preparations were iodinated with ¹²⁵I (specific activity 1.5 $\times 10^7$ Ci mg⁻¹) according to the procedure of Hunter and Greenwood¹⁶. Femoral marrow and spleen cell suspensions were mixed at 0 °C for 30 min with the ¹²⁵I-antiserum and washed twice through foetal calf serum gradients. The final concentration of antibody in the first preparation was 10 μ g ml⁻¹; that in the commercial antisera was unknown. Smear preparations were dipped in melted Kodak NTB2 emulsion and exposed for 3 d.

Two mice injected with anti-IgM from birth were killed at 3 months of age. Autoradiographs of cells exposed to ¹²⁵I-anti IgM showed that 1% or less of the small lymphocytes in the spleen were IgM bearing (> 10 grains per cell) compared with 53.3% in control spleen (Table 1). In the bone marrow, IgM-bearing small lymphocytes totalled only 0.3% or less of all nucleated cells as a result of great reductions both in the total number of small lymphocytes and in the proportion showing ¹²⁵I-anti IgM labelling (Table 1). The small number of residual bone marrow small lymphocytes may represent mainly recirculating T lymphocytes¹¹⁻¹³. The incidence in the bone marrow of large lymphoid (transitional) cells, which include the immediate precursors of small lymphocytes, was also reduced from 2.0% in the controls to 0.2% in anti-IgM-treated mice. In a separate experiment, bone marrow and spleen cells from two mice treated with anti-IgM serum for the first four weeks of life showed a similar depletion of IgM-bearing cells (mean: marrow, 0.7%; spleen, 1.1%) compared with two normal controls (mean: marrow, 39.6%; spleen, 66.4%). These results confirm the findings of a previous report, indicating depletion of Ig-bearing cells in the spleen of mice injected with anti- μ antibodies as newborn⁹, and extend them to the bone marrow.

Three irradiated, marrow-reconstituted adult mice were injected with anti-IgM for 3 months and were 22 weeks of age when studied by autoradiography. The incidence of cells bearing Ig of various classes in the spleen and bone marrow of these animals and in two groups of controls is shown in Table 2.

The results in the two groups of controls are comparable, suggesting that repopulation is complete 3 months after X irradiation and bone-marrow reconstitution. The incidence of IgM-bearing cells in the spleen of these mice is rather high when compared with other reports^{14,17}, while the number of IgA-bearing cells is lower than reported previously¹⁷. These differences may be due to differences in the age and strain of mice used, or in the reagents employed. In the bone marrow, the large proportion of IgM-bearing cells agrees with previous findings¹⁴. The incidence of small lymphocytes bearing the other Ig heavy chains has not been reported previously in mouse bone marrow. The IgG₂-bearing cells in the bone marrow are clearly more numerous than could be due to immigrant cells^{12,13}, indicating that they are part of the indigenous population of small lymphocytes maturing in the bone marrow. This supports the view that murine bone marrow has bursa-like properties. It remains to be shown whether the smaller numbers of

Table 1 IgM-bearing small lymphocytes in spleen and bone marrow of DBA/2 mice treated with anti-IgM serum from birth

Pretreatment of mice*	Spleen small lymphocytes	Bone marrow small lymphocytes	
	IgM-bearing (%)†	IgM-bearing (%)†	Total incidence (%)‡
Saline	53.3	44.0	17.4
Anti-IgM	0.3	1.0	3.4
Anti-IgM	1.0	7.4	3.5

* Three intraperitoneal injections per week from birth, until 3 months of age.

† Small lymphocytes labelled with ¹²⁵I anti-IgM per 100 small lymphocytes.

‡ Small lymphocytes per 100 nucleated cells.

Table 2 Small lymphocytes bearing immunoglobulin of various heavy chain classes in spleen and bone marrow of adult DBA/2 mice, treated with anti-IgM serum

Pretreatment of mice	Spleen small lymphocytes Ig heavy chains (%) [*]				Bone marrow small lymphocytes Ig heavy chains (%) [*]				Total incidence (%) [†]
	μ	γ_1	γ_2	α	μ	γ_1	γ_2	α	
Nil	69.5	5.5	38.0	6.5	46.0	5.3	26.5	4.7	25.8
X irradiation + BM + saline	63.0	6.0	37.5	8.5	47.0	5.7	29.5	6.0	21.8
X irradiation + BM + anti-IgM	0 [‡]	0	0	0	0.3	2.0	1.0	1.0	2.9 [5.3, 2.3, 1.1]

^{*} Labelled small lymphocytes per 100 small lymphocytes.

[†] Small lymphocytes per 100 nucleated cells.

[‡] No labelled intact small lymphocytes were seen on extensive scan.

IgG₁ and IgA-bearing lymphocytes are also produced locally in the bone marrow, or are simply part of the recirculating lymphocyte pool.

Treatment of adult X-irradiated, bone marrow-reconstituted mice with anti-IgM resulted in the virtual elimination of all Ig-bearing small lymphocytes from the spleen and bone marrow (Table 2). As with newborn mice treated with anti-IgM, the few Ig-bearing small lymphocytes detected were pycnotic in appearance and their labelling was generally near threshold (approximately 12–15 grains, compared with heavy labelling, frequently exceeding 30 grains per cell, in controls). These experiments constitute the first successful application of anti-IgM to suppress the generation of Ig-bearing lymphocytes in adult animals. It has been reported that administration of anti- μ chain antibodies does not suppress IgM synthesis in normal adult³, or 7-d-old mice¹⁸. Our results demonstrate that anti-IgM can be suppressive in the adult animal after X irradiation and bone marrow reconstitution. A full report describing the immune status of these mice will be published elsewhere (J. Gordon, *et al.*, in preparation).

The mode of action of anti-IgM and the identity of the target cell are not entirely clear. Probably, anti-IgM acts primarily on early precursors of Ig-bearing cells. Supporting this view are the present observations in the bone marrow that anti-IgM treatment eliminates "null" (Ig-ve; Thy 1-ve) small lymphocytes, previously shown to be the youngest marrow small lymphocytes¹¹, and many large lymphocyte precursor cells. The question may be raised how anti-IgM can affect these cells on which sensitive autoradiographic techniques fail to reveal Ig¹⁴. The answer may be given by recent data of Melchers *et al.*¹⁹, demonstrating a subpopulation of rapidly sedimenting marrow cells which synthesise 7–8S IgM with a considerably more rapid turnover than that of mature small lymphocytes. Conceivably, cells having such a rapid transport and release of endogenous IgM through their surface membrane may be sensitive to anti-IgM, yet apparently Ig-negative by conventional antiglobulin-binding techniques¹⁹.

Not all types of Ig-bearing cells were eliminated by anti-IgM treatment in adult mice. In both the spleen and bone marrow, well marked antiglobulin binding was still shown by a small number of plasma cells bearing IgG₁, IgG₂ and IgA, as well as monocytoïd cells, large macrophages, and damaged cells, bearing each heavy chain immunoglobulin class. Further work is required to determine the origin and significance of these cells.

The present results are consistent with the model according to which IgM-bearing cells give rise to lymphocytes carrying all other classes of immunoglobulins⁶. Murine bone marrow, like the avian bursa of Fabricius, seems to be the site of an IgM to IgG₂ sequence during lymphocyte maturation.

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Soluble factors substitute for T-T-cell collaboration in generation of T-killer lymphocytes

IMMUNOLOGICAL responses to histoincompatible tissues and to tumour cells are mediated by thymus-derived (T) lymphocytes. The cells that effect these responses have been termed cytotoxic T-killer cells and are derived from T-precursor cells that have become programmed (committed) to recognise specific antigenic determinants. The generation of T-effector or killer cells involves the collaboration of two distinct subpopulations of T cells. Helper T cells interact with specific precursor T cells when stimulated by antigen and, during the resultant differentiation processes, the precursor clones mature into specific killer cells^{1–3}. The nature or mechanisms of the collaboration between the precursor and helper T cells has been obscure until now. The data presented here demonstrate that soluble T-cell products can replace the requirement for helper T cells in the generation of T-killer cells. The active substances are produced on antigenic stimulation and are as effective as T-helper cells during the differentiation of precursor T cells into killer cells.

Antigens of the murine major histocompatibility complex, H-2, were used. H-2-associated antigens served both as the stimuli for the precursor and helper T cells and as targets for the immunologically activated cells. An antigen associated with the H-2 complex also served as a cell-surface marker for a helper

Table 1 The effects of anti- θ and anti-Ha serum treatments on responding cells in mixed lymphocyte cultures

Pretreatment of C57BL/10 responding cells*	Stimulating cells (1,000 rad)			
	None	B10.A(2R)	B10.A(5R)	B10.D2
	Average c.p.m. of ^3H -thymidine incorporation $\pm$ s.e.			
NMS+RC	489 $\pm$ 83	13,291 $\pm$ 735	5,138 $\pm$ 1,065	22,808 $\pm$ 2,613
Anti-Ha†+RC	213 $\pm$ 19	2,655 $\pm$ 582	570 $\pm$ 88	1,906 $\pm$ 64
Anti- θ ‡+RC	620 $\pm$ 48	2,285 $\pm$ 554	905 $\pm$ 153	1,591 $\pm$ 167
Anti- θ +RC—4 $\times$ 10 ⁶ per ml	Mixture of pretreated cells	2,575 $\pm$ 558	817 $\pm$ 240	3,852 $\pm$ 1,169
Anti-Ha+RC—6 $\times$ 10 ⁶ per ml				
No. of responding cells		102 $\pm$ 4	150 $\pm$ 13	107 $\pm$ 12

* A total of 10⁶ viable and washed cells were cultured with an equal number of stimulating cells for 98 h including an 18-h pulse with 0.5 μCi ^3H -thymidine per ml. Procedural details are outlined in Table 2.

† B10.A anti-B10.D2 serum plus rabbit serum as a source of complement.

‡ (AKR $\times$ RF)₁ anti-C58 thymus cells.

T-cell subpopulation. The precise *H-2* genetic region coding for the T-helper cell antigen is under investigation. The helper cell antigen is temporarily designated Ha.

The anti-helper cell serum (anti-Ha) was raised in B10.A mice against B10.D2 lymph node cells (LNCs). These congenic resistant partners differ at *H-2K* and portions of the I region of

responses and the development of killer cells to allogeneic *H-2* antigens were assessed with C57BL/10 LNCs that had been treated, before culturing, with either normal mouse serum (NMS), B10.A anti-B10.D2 serum (anti-Ha) or anti- θ serum (Thy-1.2) plus complement (RC). Rabbit serum extensively absorbed with mouse tissues served as the source of comple-

Table 2 Effect of supernatant factors on killer-cell development in cultures containing C57BL/10 cells pretreated with NMS, B10.A anti-B10.D2 (anti-Ha) serum or anti- θ serum plus complement and mixed with irradiated B10.D2 cells

Serum pretreatment of responding cells		Medium % ^{51}Cr release†	Supernatant	Medium Total killing activity‡	Supernatant
Experiment A§	Anti-Ha+RC	17.1	52.8 $\pm$ 2.5	11	169
	NMS+RC	48.0 $\pm$ 3.3	58.7 $\pm$ 2.0	145	153
Experiment B¶	Anti- θ +RC	11.4 $\pm$ 0.5	11.8 $\pm$ 0.1	1	5
	NMS+RC	55.0 $\pm$ 0.0	55.9 $\pm$ 1.2	248	267

* Either 1.0 ml of fresh medium or of diluted supernatant from activated C57BL/10 LNCs stimulated by B10.D2 cells was added to each culture on day 0. The final concentration of supernatant was 21% in experiment A and 17% in experiment B.

† Spontaneous ^{51}Cr release of P815-X2 target cells after 4 h was 7.7% $\pm$ 0.2 in experiment A and 10.7% $\pm$ 0.2 in experiment B. Data are presented as % $\pm$ s.e.

‡ Total killing activity = % specific ^{51}Cr release $\times$ cell yield after 5 d in culture per 10⁶ cells.

§ 1.0 ml of diluted serum (1:5) was added to a pellet of 15 $\times$ 10⁶ C57BL/10 LNCs and incubated in a shaking water bath at 37 °C for 15 min. An equal volume of diluted RC (1:4) was added and the suspensions were incubated for a further 45 min. Antiserum and RC were removed by centrifugation and the cells washed twice in fresh medium. Equal numbers of remaining viable cells and irradiated B10.D2 cells (2 $\times$ 10⁶ per 0.5 ml) were then cultured in RPMI 1640 medium supplemented to 0.01 M HEPES buffer, 8% human serum (heated at 56 °C for 30 min), 100 U penicillin G, 150 μg L-arginine, 2 mM L-glutamine and 75 μg kanamycin sulphate per ml.

¶ A sample of 1.0 ml of diluted serum (1:5) was added to a pellet of 4 $\times$ 10⁷ C57BL 10 per LNCs and treated further as above before mixing with irradiated B10.D2 LNCs.

the *H-2* complex. Anti-Ha serum greatly depresses the T-cell responses of C57BL/10 LNCs to foreign *H-2* antigens, *in vitro*⁴ (Table 1). The effectiveness of the anti-Ha serum against the responding cell population is demonstrated by the elimination of a response to B10.A(2R)-stimulating cells (Table 1). B10.A (2R) cells are insensitive to lysis by the B10.A anti-B10.D2 serum and share *H-2K* and I-region specificities with the B10.A antibody producer⁵. Although a proportion of the cytotoxic activity to C57BL/10 cells is directed against an I-region antigen on B cells⁵, depletion of non-T cells is not responsible for the depressed T-cell activity, as the mixed lymphocyte responses (MLRs) could not be restored by addition of syngeneic B cells (Table 1) in increasing numbers or of macrophages⁴. Cell-free factors produced by activated T cells could, however, reconstitute the ability to respond.

The soluble helper factor(s) was obtained through a two-step tissue culture procedure. C57BL/10 LNCs were first sensitised in culture for 4 or 5 d against irradiated B10.D2 strain LNC. The activated cells were then washed and mixed with an equal number of fresh stimulating strain LNCs. The cell mixtures (4 $\times$ 10⁶ of each) were cultured in 1.0 ml of RPMI 1640 medium without serum, centrifuged at 200g for 5 min and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Six hours later the cells were resuspended, pooled and centrifuged at 900g for 10 min. To ensure removal of all cells, the supernatant fluid was filtered through a 0.45- μm Millipore filter. The supernatants were either diluted and used immediately or frozen at -70 °C in the presence of 10% human serum.

The effects of the supernatant factor(s) on proliferative

ment⁷. Anti- θ serum was raised in (AKR $\times$ RF)₁ mice against C58 strain thymus cells. The effects of this anti- θ serum on the MLRs were reported previously⁸.

The generation of cytotoxic killer T cells was greatly reduced in cultures of cells that remained after lysis with either anti-Ha serum or anti- θ serum plus RC (Table 2). The addition of the supernatant factor to the antibody-treated cells completely reversed the depressive effect of the anti-Ha serum but had no effect on killer cell development in cultures with anti- θ treated cells (Table 2).

Table 3 Effect of supernatant factors on the proliferative responses of C57BL/10 cells pretreated with either NMS, B10.A anti-B10.D2 serum (anti-Ha) or anti- θ serum plus RC

Serum pretreatment of responding cells	Supernatant concentration		
	0%	25%	6.25%
	Average c.p.m. of ^3H -thymidine incorporation $\pm$ s.e.*		
Anti-Ha+RC†	1,889 $\pm$ 636	11,495 $\pm$ 304	10,108 $\pm$ 53
NMS+RC†	42,353 $\pm$ 5,859	46,494 $\pm$ 1,594	41,069 $\pm$ 735
Anti- θ +RC†	6,022 $\pm$ 773	8,367 $\pm$ 982	8,530 $\pm$ 371

* The cultures were collected after 87 h which included a pulse of 0.5 μCi ^3H -thymidine per ml during the final 19 h.

† The background c.p.m. of the serum treated cells were as follows: NMS+RC, 7,696; anti-Ha serum+RC, 146, and anti- θ serum+RC, 445.

C57BL/10 LNCs were treated with antiserum plus complement as outlined in Table 2. Equal numbers of viable serum-treated cells and irradiated B10.D2 cells (1.5 $\times$ 10⁶ per 0.5 ml) were mixed. 1.0 ml of either fresh medium or of diluted supernatants from C57BL/10 activated cells was then added to triplicate cultures of each combination.

Table 4 Effect of supernatant factors on the proliferative responses of non-stimulated and MLR-stimulated cell cultures

Cells cultured	Supernatant concentration*				
	0%	50%	25%	12.5%	6.25%
C57BL/10	2,253 ± 33	(average c.p.m. of ³ H-thymidine incorporation ± s.e.)† 3,240 ± 571	2,585 ± 187	3,473 ± 446	2,325 ± 123
C57BL/10 + B10.D2 (1,000 rad)	11,852 ± 1,852	15,581 ± 549	18,749 ± 1,092	15,341 ± 1,415	10,065 ± 1,059
B10.D2 + C57BL/10 (1,000 rad)	8,875 ± 1,883	18,148 ± 3,610	13,897 ± 1,069	16,015 ± 1,069	5,814 ± 1,264

* The supernatant used in these cultures was produced by C57BL/10 cells after activation with B10.D2 irradiated cells for 5 d.

† The cultures were collected after 66 h which included a pulse of 0.5 µCi ³H-thymidine per ml during the final 18 h of culture.

Three conclusions can be drawn from these data. First, the C57BL/10 precursor T cells that respond to H-2 antigens are not lysed by the anti-Ha serum plus RC. We have also demonstrated that the depressive effect of this antiserum on MLR cannot be reversed by the addition of various numbers of cells remaining after lysis with anti-θ serum plus RC⁴ (Table 1). Therefore, the depleted H-2 responsive cell must reside in the θ-antigen-bearing T-cell population but not, as demonstrated in Table 2, in the precursor subpopulation. The most likely target of the B10.A anti-B10.D2 serum, then, is the helper T cell.

Second, the supernatant factor(s) can serve functionally as an adequate substitute for the antibody-depleted cells in the generation of killer T cells. Although much information has been accumulated about T-cell replacement factors in B-cell-mediated responses⁹⁻¹⁸, this is the first evidence that a stable soluble helper product mediates T-T-cell collaboration.

Third, one of the functions of the helper factor(s) is to increase the numbers of killer cells generated. The total yield of killer cell activity obtained from C57BL/10 cells treated with the anti-Ha serum and subsequently cultured with stimulating cells in the presence of the supernatant factor(s) was equivalent to that obtained with NMS-treated cells (Table 2). This increase in killing activity was probably accomplished through the enhancement of clonal proliferation of the precursor cells. Clearly, the helper factor provided a signal that was necessary for DNA synthesis (Table 3). The helper factor was active even at quite dilute concentrations, in the enhancement of the MLRs obtained with cells remaining after lysis with anti-Ha serum (Table 3). The helper factor(s) had only slight effects on the proliferative responses of cultures that either contained both (NMS + RC) or lacked both (anti-θ + RC) the precursor and helper T-cell subpopulations (Table 3). Similar results were obtained with C57BL/10 LNCs cultured without irradiated stimulating cells (Table 4). Enhanced proliferation of unstimulated cells was never greater than 2.5-fold over background in three separate experiments. The helper factor did enhance MLR-induced proliferation within a wide range of concentrations and apparently was not specific for the stimulating H-2 antigens (Table 4).

The difference between the levels of thymidine incorporated and the total yield of cytotoxicity generated in supernatant-supplemented cultures of cells remaining after lysis by anti-Ha serum is revealing. In the normal MLR, helper cells as well as precursors proliferate, thus both subpopulations contribute to the observed responses. The proliferative contribution of helper cells is lost, however, in cultures pretreated with anti-Ha serum. At the levels of supernatant tested, maximum ³H-thymidine incorporation by the antiserum-resistant precursor cells is reached even at the lower concentrations (Table 3). The total yield of killer-cell activity in the supernatant-supplemented cultures of anti-Ha-treated cells is equivalent to that obtained with unfractionated T-T-cells (Table 2). Thus the sequence of events in the generation of T-effector cells seems to be: the release of soluble factors and proliferation of helper cells on contact with antigen and the subsequent stimulation of proliferation and potentiation of specific precursor cells by the helper factor and antigen.

The relationship between the helper factor described here and the T-cell replacement factors that function in mediating B-cell responses is being investigated. Many of the factors that help in antibody production have been obtained from allogeneic cell cultures. The nonspecific allogeneic effect factor¹⁷ and the specific

T-cell factor of Munro and Taussig¹⁸ which mediate B-cell responses are of particular interest as each can be removed with anti-H-2 serum-conjugated immunoabsorbants. It is possible that the H-2-associated marker detected on the helper T cells is similar or identical to a determinant on the soluble helper substance probably produced by these cells. This helper cell marker is expressed by a small proportion of T-effector cells and the end-stage or mature killer cells may no longer express the antigen⁴. This supports the notion that the H-2-associated helper cell marker is also expressed by the soluble helper product and that it functions in the differentiation of the precursor T cells to effector T cells.

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Pharmacological effects of phosphatidylserine liposomes

PHARMACOLOGICAL studies on diacylphospholipids develop along two lines of investigation. In the first, preparations of liposomes are used as carriers of drugs either bound to the bilayer or entrapped inside the vesicles. Sonicated liposomes have been shown to be able to transport inhibitors or drugs to target sites *in vitro*¹ and *in vivo*². In the second, liposome preparations are administered and pharmacological effects are recorded. In this context significant effects have been observed on cholesterol distribution³. The molecular mechanism underlying the possibility of pharmacological effects *in vivo* by long chain diacylphospholipids is indicated by a series of investigations⁴⁻⁷ showing the capacity of phospholipid vesicles to fuse with cellular membrane *in vitro*. As a consequence of changed composition of membrane lipids, alteration in the permeability properties, transport systems and other functions of biological membranes are expected to occur. It has been shown⁸ that sonicated dispersion of bovine brain phospholipids can induce in mice modification of glucose distribution. Purifica-

tion of lipid mixtures indicated that phosphatidylserine was one of the active components. Phosphatidylserine liposomes have shown great capability to interact *in vitro* with biologically active compounds (histamine⁹ or calcium¹⁰), with phospholipid-dependent enzymatic systems (Na^+ - K^+ -stimulated ATPase¹¹ or tyrosine hydroxylase¹²), or with intact cells (enhancement of histamine release during antigen-antibody interaction¹³; promotion of cell fusion⁴). In this investigation data on phosphatidylserine-induced effects *in vivo* are presented.

Fed male albino mice were used throughout. The purity of phosphatidylserine extracted from bovine brain¹⁴ was checked by thin-layer chromatography using Silica gel G and a solvent of chloroform-methanol-water (65:35:4, by volume). Dispersion of phosphatidylserine was obtained by 8-min sonication (MSE apparatus) at 0 °C in 50 mM Tris-HCl, pH 7.4. Liposomes in 0.1–0.2 ml buffer were injected intravenously in the tail vein within a few hours of their preparation, and the animals killed 30 min later by decapitation so as to collect the blood and allow the head to fall into liquid nitrogen. Brain hemispheres were removed, powdered under liquid nitrogen together with frozen 0.5 ml 0.3 N perchloric acid and allowed to warm at 0–4 °C. Glucose was estimated by the hexokinase method on perchloric acid extract¹⁵.

Figure 1 shows the effect of phosphatidylserine liposomes on free glucose content of blood and brain tissue. The level of glucose was increased in both compartments; 15–30 mg kg⁻¹ were required to trigger the effects (0.35–0.70 mg per mouse, equal to about 0.5–1.0 μmol phosphatidylserine). This low amount, which can be calculated to be composed of about 2×10^{14} vesicles⁴, is not expected to produce gross changes in the body circulation. Accordingly, variations in brain glycogen or lactate were not recorded. Parallel experiments showed that neither uncharged liposomes of phosphatidylcholine nor charged, small liposomes of bovine brain phosphatidylinositol produced effects on glucose distribution. Among phosphatidylserines, liposomes made by purified soya bean phosphatidylserines were only slightly or not active, suggesting a role of both acyl chain and structure of polar head. As it was observed with the mixture of bovine brain phospholipids⁸, the effect of phosphatidylserine liposomes was more manifest after intravenous

Fig. 1 Effect of increasing amounts of phosphatidylserine on glucose distribution in mice. Sonicated dispersion of liposomes in 50 mM Tris-HCl (pH 7.4) were injected intravenously to fed mice which were killed 30 min later. Other conditions described in text. Values are mean $\pm$ s.e. of four animals. ●, Blood; ■, brain.

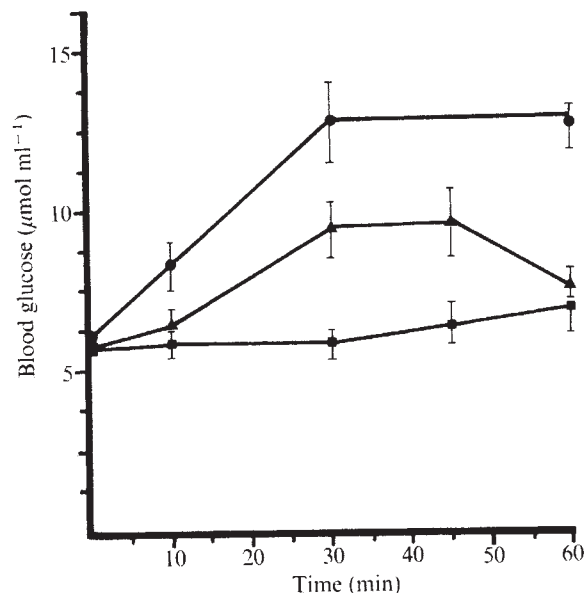
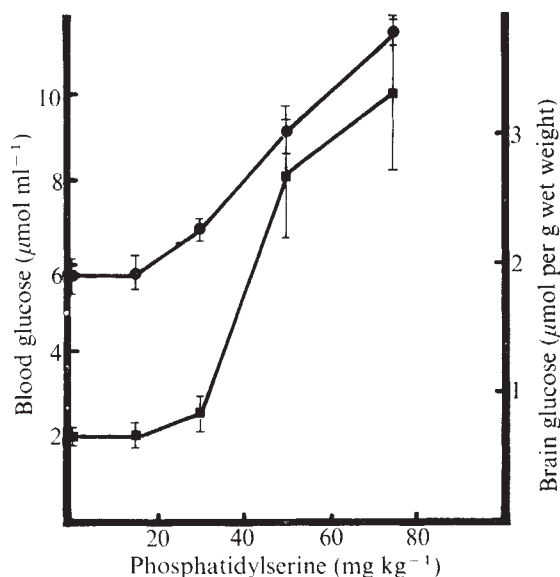


Fig. 2 Antagonism by Hydergine (dihydroergotoxine): sonicated liposomes (75 mg kg⁻¹) containing phosphatidylserine were given intravenously to mice treated (▲) or not (●) with hydergine (2 mg kg⁻¹) 30 min before phospholipid injection. Mean $\pm$ s.e. of four animals. ■, Hydergine alone.

injection and dispersion by sonication at neutral pH (Table 1). The enhancement of brain-blood glucose ratio revealed the tendency for glucose accumulation in the brain tissue. The need for small liposome formation in order to produce the pharmacological effect is difficult to explain in terms of possibility to cross membrane barriers. The average diameter of phosphatidylserine liposomes prepared by sonication¹⁶ is estimated to be about 200 Å, not compatible with free transfer across cellular membranes, the blood-brain

Fig. 3 Decrease in adrenal catecholamine content. Mice were injected intravenously with sonicated liposomes of phosphatidylserine (150 mg kg⁻¹). At the time indicated the animals were killed and the adrenals homogenised in 0.4 N perchloric acid containing 0.1% sodium metabisulphite. Total catecholamines determined¹⁸ on about 2 mg tissue. Values are mean $\pm$ s.e. of separate determinations whose number is given in parentheses.

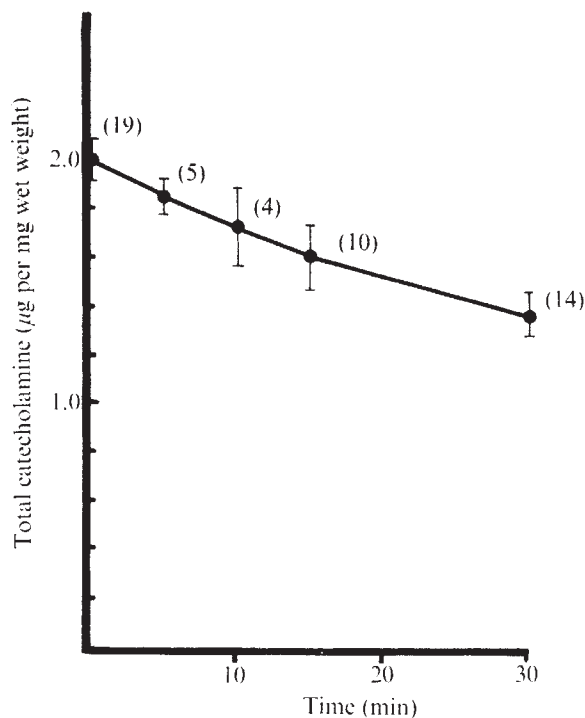


Table 1 Effect of phospholipid dispersion

Liposomes dispersed by	Administration	Blood glucose ($\mu\text{mol ml}^{-1}$)	Brain glucose ($\mu\text{mol per g wet weight}$)	Brain-blood ratio
—	—	6.21 $\pm$ 0.13	1.33 $\pm$ 0.05	0.21
Homogenisation (17)	Intraperitoneal	6.76 $\pm$ 0.67	1.44 $\pm$ 0.05	0.21
Sonication (5)	Intraperitoneal	7.45 $\pm$ 0.59	1.62 $\pm$ 0.05*	0.22
Homogenisation (5)	Intravenous	7.35 $\pm$ 0.79	1.55 $\pm$ 0.18	0.21
Sonication (17)	Intravenous	10.17 $\pm$ 0.40*	3.67 $\pm$ 0.23*	0.36

* $P < 0.001$.

Liposomes were injected into fed mice at 75 mg phosphatidylserine per kg. Dry phospholipid was resuspended in 50 mM Tris-HCl buffer and dispersed by simple homogenisation or sonication (see text). Number of animals given in parentheses. Values are mean $\pm$ s.e. Significance levels (P) were assessed using Student's t test.

barrier or even the capillary wall of many organs. The effect of sonication is better understood in terms of facilitation of lipid-lipid, lipid-protein interaction or binding of blood cations. Penetration of liposomes into parenchymal cells is also enhanced after reduction of their size by sonication¹⁷. This would enable liposomes to produce a direct influence on cell carbohydrate metabolism in some organs (for example, liver). In spite of its high activity, the phosphatidylserine content does not completely account for the effect of bovine-brain phospholipid mixture observed earlier⁸. It is possible that other active components are present or that the optimal ratio between phosphatidylserine and other phospholipids increased the effect. A mixture of phosphatidylcholine, lysophosphatidylcholine and phosphatidylserine was more active than phosphatidylserine alone in inducing cell fusion⁴.

The involvement of catecholamine release in the hyperglycaemic effect is indicated in the experiments of Fig. 2 in which it is shown that previous administration of the adrenolytic agent Hydergine (dihydroergotoxine) strongly reduced the phosphatidylserine-induced increase of blood glucose level. In separate experiments it was found that in the Hydergine-treated mice the normal values of glycaemia did not prevent the effect of liposomes on brain glucose, in agreement with the observations on lipid mixtures⁸. Attempts were made to demonstrate a decreased level of catecholamine content in the adrenal glands after phosphatidylserine injection. Figure 3 shows that a reproducible reduction of about 30% was found 30 min after administration of phospholipid vesicles (150 mg kg⁻¹). The increased cerebral content of glucose, the lack of lactate accumulation and the ineffectiveness of phosphatidylcholine and phosphatidylinositol excluded the possibility that nonspecific damage of blood circulation followed by cerebral hypoxia was responsible for catecholamine release.

The accumulation of free glucose in brain tissue and the release of catecholamines are phosphatidylserine-induced pharmacological effects which can be differentiated by the use of an adrenolytic agent. In spite of this distinction, a correlation between the two effects seems likely and it is indicated by the observation of a dopamine-induced high level of brain glucose¹⁹. The same pattern of activity (ineffectiveness of phosphatidylcholine and phosphatidylinositol, high activity of phosphatidylserine) was found on histamine release *in vitro*²⁰, an effect which required calcium. Calcium bridges between the negatively charged cell surface and liposomes may be required for the attachment of vesicles, as was suggested for phosphatidylserine-induced fusion of cultured cells⁴. This indicates a general capacity of phosphatidylserine vesicles to promote *in vivo* and *in vitro* the calcium-dependent release of cellular materials stored in specialised structure. Extending these observations it was found that injection of sonicated liposomes of bovine brain phospholipids increased catecholamine metabolism in mouse brain²¹ and produced release of acetylcholine from rat brain cortex²². Phosphatidylserine was the active component in both cases. Further investigations are

required to establish whether these effects taking place in the central nervous system reflect liposome penetration into the brain fluids.

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Bacterial membrane transport of β -exotoxin, an anti-metabolite of RNA synthesis

β -EXOTOXIN is produced extracellularly by certain strains of *Bacillus thuringiensis* during the stationary growth phase, apparently resulting from nucleic acid metabolism¹. It is a monophosphorylated adenine nucleoside derivative^{2,3} and can modify RNA polymerase activity of mammalian and bacterial origin *in vitro* through substrate competitive inhibition^{4,5}. RNA polymerase in nuclei from rat liver⁶ and insect larval fat bodies⁷ is sensitive to exotoxin, and inhibition seems to be subject to reversal by ATP. Other functional enzymes that catalyse reactions involving ribonucleoside triphosphates are similarly inhibited by exotoxin, such as adenylate cyclase⁸. Exotoxin is more toxic in mammalian and other eukaryotic systems than in cell-free prokaryotic extracts, and it can cross cell and nuclear

membranes in mammalian systems^{5,6,9}. But I know of no report of any effect of exotoxin on intact, physiologically normal bacterial cells. Therefore I have investigated the intracellular effect of exotoxin on RNA synthesis in actively metabolising bacterial cultures. This report describes the intracellular inhibition of RNA synthesis by exotoxin in cultures of *Escherichia coli* and *Bacillus subtilis* treated to increase their permeability. Further data revealed that exotoxin is totally excluded by the bacterial membrane, even in those treated cells.

Both organisms were impermeable to exotoxin in normal conditions. Extracellular exotoxin concentrations up to 1 mg ml^{-1} caused only slight inhibition (5% or less) of RNA synthesis in exponential cultures of *B. subtilis* and *E. coli*. Initially, no methods designed to increase membrane permeability of exponentially growing cells¹⁰⁻¹³ increased exotoxin inhibition of cellular RNA

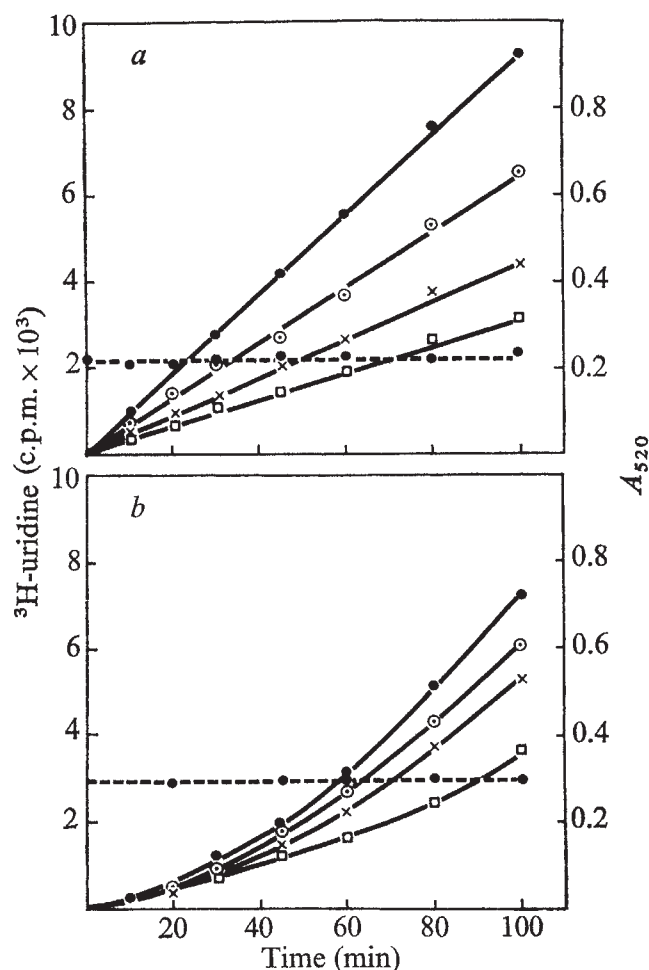


Fig. 1 *In vivo* inhibition of RNA synthesis by exotoxin. *a*, *E. coli*; *b*, *B. subtilis*. Cultures were pretreated by the EDTA-pH shift method. Extracellular exotoxin concentration: ●, $0 \text{ } \mu\text{g ml}^{-1}$; ○, $50 \text{ } \mu\text{g ml}^{-1}$; ×, $100 \text{ } \mu\text{g ml}^{-1}$; and □, $500 \text{ } \mu\text{g ml}^{-1}$. The absorbance of an uninhibited control culture (without exotoxin) is also shown (---). Cultures of *E. coli* and *B. subtilis* were grown on a minimal salts medium at pH 6.8 supplemented with 0.2% glucose; filter-sterilised tryptophan ($40 \text{ } \mu\text{g ml}^{-1}$) was added for growth of the *Bacillus* culture. Mid-exponential phase cultures were treated to increase their permeability by Leive's method¹⁰. Treatment was for 2 min (25°C) with 0.25 M EDTA, after which the culture was diluted with 0.125 M Tris-HCl, centrifuged, washed again with Tris-HCl, recentrifuged, and the pellet resuspended in complete minimal medium at pH 5.2. Measurement of isotope incorporation in these permeabilised cells was determined by incubation on a water bath shaker (30°C) in the presence of ^3H -uridine (specific activity, $3.7 \text{ } \mu\text{Ci } \mu\text{mol}^{-1}$) and various concentrations of exotoxin by withdrawing 1-ml samples at intervals and by precipitating the RNA with cold 10% TCA. The samples were filtered on Whatman GF/A glass-fibre filters, washed with TCA (containing 0.4 mM carrier uridine) and cold ethanol, dried, and counted by liquid scintillation.

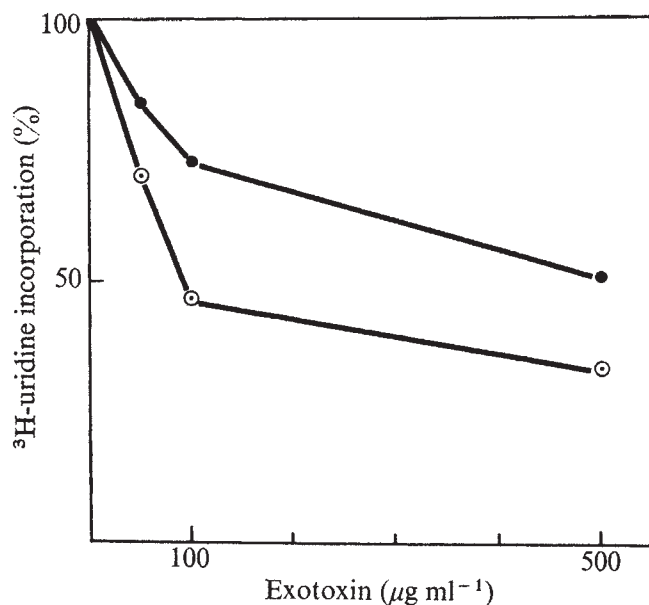


Fig. 2 Percentage inhibition of ^3H -uridine incorporation by exotoxin. ○, *E. coli*; ●, *B. subtilis*. Values are percentage incorporation compared with an uninhibited control after 100 min incubation, and reflect the final data points from Fig. 1 for both organisms.

synthesis. An adaptation of one method, however, involving EDTA-Tris treatment followed by resuspension of the washed cell pellet in complete minimal medium at pH 5.2, gave greater sensitivity to exotoxin. The rate of RNA synthesis was near normal in these cultures, although a lag of 2-3 h occurred before replication resumed.

Incorporation of ^3H -uridine into trichloroacetic acid (TCA)-precipitable RNA in the presence and absence of exotoxin is shown in Fig. 1. Total RNA synthesis after 100 min in *E. coli* cells treated with EDTA-Tris was inhibited 29.9, 53.2 and 66.1%, respectively, by the addition of exotoxin at 50, 100 and $500 \text{ } \mu\text{g ml}^{-1}$. Corresponding data were obtained from *B. subtilis* cells pretreated similarly; inhibition of total RNA synthesis after 100 min was 16.3, 23.1 and 49.2%, respectively, in the presence of the same concentrations of exotoxin. No cell division occurred with either EDTA-Tris-treated culture during the measured interval of uridine incorporation.

The degree of inhibition at each concentration of exotoxin fluctuated little throughout the experiment with both organisms. The inhibitory response, however, was not linear with respect to increased exotoxin concentration, but became less sensitive above $100 \text{ } \mu\text{g ml}^{-1}$, or $1.25 \times 10^{-4} \text{ M}$ (Fig. 2). These results corroborate data for *in vitro* exotoxin inhibition for purified *E. coli* RNA polymerase¹⁴.

Attempts to measure exotoxin transport into EDTA-Tris-treated cells (not pH shifted) of either organism failed. The uptake of ^3H -uridine and ^3H -exotoxin was measured in the presence of rifampin so that only membrane transport would be measured and incorporation could be excluded. The effect of unlabelled exotoxin on ^3H -uridine uptake was followed. Exotoxin did not penetrate the membrane of EDTA-Tris-treated cells of either *E. coli* (Fig. 3) or *B. subtilis*. Uridine transport was most rapid during the first 5-10 min in both organisms, and then it diminished to a plateau when an inhibitor of RNA synthesis was present. Unlabelled exotoxin had little effect on uridine transport of *B. subtilis*, but slightly reduced uridine transport in *E. coli*. Growth was resumed in EDTA-Tris-treated cultures approximately 20 min after treatment.

Historically, bacterial membranes have been known to be poorly permeable to hydrophilic compounds. Nucleotides and hexose phosphates are classic examples. EDTA treatment disrupts the outer membrane of Gram-negative bacteria and provides improved access for some compounds, yet the inner

membrane remains intact and presents still another barrier for certain external molecules¹⁵. Whereas deoxyribonucleoside triphosphates gain rapid entrance inside permeabilised cells of *E. coli*, the corresponding monophosphates are almost totally excluded¹⁶. The exotoxin produced by *B. thuringiensis* is structurally similar to deoxyribonucleoside monophosphates, and its inhibitory activity toward RNA polymerase is based on substrate analogue competition in cell-free extracts. My results, however, suggest that whatever the effect of exotoxin on cellular RNA synthesis in bacteria, it occurs externally. Figure 3 indicates that exotoxin may depress uridine uptake in *E. coli*, which could interfere with normal internal ribonucleotide precursor pools. Comparable experiments with cells of *B. subtilis* were inconclusive.

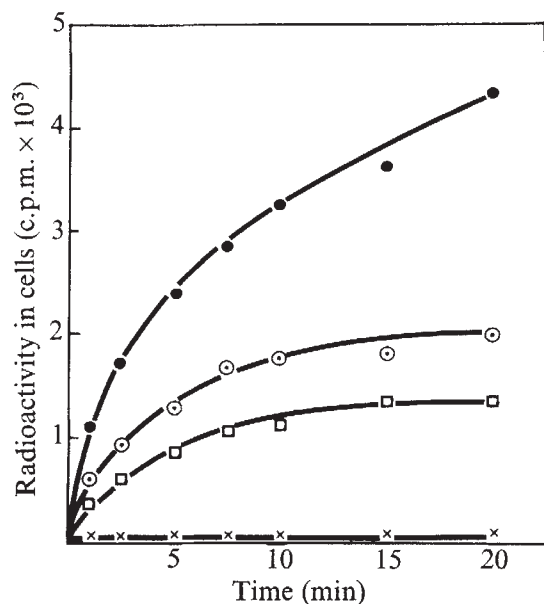


Fig. 3 Uptake of ³H-uridine and ³H-exotoxin in cells of *E. coli* treated with EDTA-Tris. ³H-uridine uptake: ●, without rifampin; ○, with rifampin (50 μg ml⁻¹); □, with rifampin and unlabelled exotoxin (100 μg ml⁻¹). ×, ³H-exotoxin uptake. Transport was measured with EDTA-Tris-treated cells, which were resuspended in complete minimal medium at pH 6.8 and held on ice until used. Either tritium-labelled uridine (Fig. 1) or exotoxin (specific activity about 0.27 μCi μmol⁻¹) was added to samples of permeabilised cells and placed in a reciprocating water bath shaker at 18 °C. Rifampin was added (50 μg ml⁻¹) to prevent *in vivo* ³H-uridine incorporation into RNA. Samples (1 ml) containing whole cells were withdrawn at intervals and immediately filtered on GF/A filters, washed with cold buffer (10 mM Tris-HCl, 50 mM KCl, pH 7.9), dried, and counted by liquid scintillation. Both labelled and unlabelled exotoxin were provided by T. R. Shieh (International Minerals and Chemical Corporation).

B. thuringiensis exotoxin has little or no effect on RNA synthesis in untreated cultures of *E. coli* and *B. subtilis*. Even in specially treated cultures with increased permeability, exotoxin seems to be totally excluded by the bacterial membrane. But RNA synthesis in similarly treated cultures of these organisms is inhibited by exotoxin in a concentration-dependent order. Thus inhibition of RNA synthesis by exotoxin in intact bacteria seems to be linked to membrane transport and not to competitive inhibition of RNA polymerase.

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Role of mitochondria in control of calcium content of liver slices

INTRACELLULAR Ca²⁺ is thought to be important in the control of metabolic activity^{1,2}. In liver cells, three energy-dependent transport activities capable of modifying the cellular distribution of this ion have been described—its accumulation by mitochondria^{3,4} and endoplasmic reticulum⁵ and its extrusion from the cell^{6,7}. Mutual interactions, however, between these activities and their relative importance in determining total cell Ca²⁺ have so far been little studied in intact cells. One reason for this has been the difficulty of avoiding redistribution of Ca²⁺ between organelles when the cells are broken for isolation procedures, although Chen *et al.*⁸ used ruthenium red, added to the homogenisation medium, to prevent such redistribution in crab hepatopancreas. We now show that redistribution of Ca²⁺ into the mitochondria of rat liver can also be largely prevented by adding La³⁺ (another inhibitor of mitochondrial Ca²⁺ transport; ref. 9) to the homogenisation medium. We have also studied the contribution of mitochondria to the Ca²⁺ content of rat liver slices incubated in conditions in which total cell Ca²⁺ varies greatly.

Slices of rat liver were prepared and incubated as described previously⁷. Mitochondria were isolated from fresh liver and from incubated slices¹⁰ using medium containing: mannitol 250 mM, sucrose 75 mM, EDTA 0.1 mM and Tris-HCl (pH 7.4) 2.5 mM. The swollen mitochondria of the 'fluffy layer' occurred in greater quantities in incubated slices than in fresh liver¹¹; as we wished to determine the total mitochondrial contribution to the tissue Ca²⁺, we usually analysed the mitochondrial pellet and 'fluffy layer' together. Mitochondria from all preparations showed coupled, oligomycin-sensitive respiration¹¹. For analysis, samples of slices and tissue fractions were dried and ions (including ⁴⁵Ca) extracted from the dry tissue⁷. Total Ca²⁺ was determined by atomic absorption spectrophotometry⁷ and ⁴⁵Ca by standard techniques of scintillation counting. Protein was determined by a Biuret method¹².

To study the effectiveness of adding La³⁺ to the homogenisation medium as a means for preventing the redistribution of Ca²⁺, we measured the entry of ⁴⁵Ca into mitochondria isolated from liver slices that had been incubated for 90 min at 1 °C. In these conditions the slices show the highest total Ca²⁺ content (see below). To half of the slices, ⁴⁵Ca was added in the homogeniser tube only 5 s before their disruption, so that the isotope could be considered totally 'exogenous'. In this case, the ⁴⁵Ca and La³⁺ present in the homogenisation medium would have reached the mitochondria simultaneously and a maximal possible inhibition of ⁴⁵Ca entry would be expected. Table 1 shows that about 50% of the added ⁴⁵Ca was taken up by the control mitochondria (homogenised without La³⁺), and that the presence of 1 mM La³⁺ inhibited this uptake by 90%. Thus, La³⁺ can be used effectively to block redistribution of tissue Ca²⁺ into the mitochondria on

Table 1 Effect of presence of La^{3+} during homogenisation on entry of ^{45}Ca into mitochondria isolated from liver slices

^{45}Ca added	Slice group	La^{3+} added to homogeniser (mM)	^{45}Ca in mitochondria ($10^{-6} \times \text{d.p.m. per g protein}$)	Total Ca^{2+} in mitochondria (mmol per kg protein)
To homogeniser	<i>a</i>	0	$35.8 \pm 4.0^*$	—
	<i>b</i>	1	3.9 ± 0.5	—
To incubating slices	<i>c</i>	0	$1.49 \pm 0.08^\dagger$	63.2 ± 2.1
	<i>d</i>	1	0.68 ± 0.05	47.3 ± 1.7

* $51.2 \pm 3.5\%$ of radioactivity in whole homogenate.

† $60.9 \pm 3.8\%$ of radioactivity in whole homogenate.

Slices were incubated for 90 min at 1°C in a Ringer solution containing, 146 mM Na^+ , 5 mM K^+ , 1.3 mM Ca^{2+} , 1.0 mM Mg^{2+} , 161 mM Cl^- , 1.0 mM SO_4^{2-} and 10 mM Tris (pH 7.4). After incubation the slices were separated from the medium by suction and washed once with the mitochondrial isolation medium to remove superficial ions. The slices were then rapidly transferred to the isolation medium. The slices were divided into four groups which were treated as follows: *a*, ^{45}Ca was added to the isolation medium in the homogeniser, to give $0.5 \mu\text{Ci ml}^{-1}$ ($0.5 \mu\text{Ci per } \mu\text{mol Ca}^{2+}$), 5 s before homogenisation of the slices. *b*, As for group *a*, but with 1 mM La^{3+} present in the homogenisation medium. *c*, ^{45}Ca was added to the Ringer solution containing the slices after incubation at 1°C for 89 min, giving $0.5 \mu\text{Ci ml}^{-1}$ ($0.38 \mu\text{Ci per } \mu\text{mol Ca}^{2+}$). *d*, As for group *c*, but with 1 mM La^{3+} present in the homogenisation medium. The medium used for rehomogenisation of the 600g pellets (ref. 9) of groups *b* and *d* contained 0.5 mM La^{3+} ; further steps in the isolation procedure were carried out in medium free of La^{3+} . The counting rate for ^{45}Ca was corrected for background, and for quenching by the channels-ratio method; counting efficiency was approximately 55%. The results reported are mean $\pm$ s.e. of the mean from four experiments, each analysed in triplicate.

homogenisation. In the remaining slices we determined the effect of La^{3+} present during homogenisation on the distribution of ^{45}Ca added 1 min before the end of incubation at 1°C (and approximately 1.5 min before homogenisation). The slices were rinsed free of the superficial, labelled Ringer solution before homogenisation so that all the remaining ^{45}Ca in the slices could be considered 'endogenous'. Mitochondria isolated from these slices without added La^{3+} again contained much of the total ^{45}Ca in the homogenate, but the presence of 1 mM La^{3+} reduced the recovery of isotope in the mitochondria by about 55%. We conclude that this inhibition largely represents the prevention of Ca^{2+} entry into the mitochondria on disruption of the tissue, and that the 45% of the ^{45}Ca still found in the mitochondria had entered by exchange before homogenisation.

It follows that the extent to which cell Ca^{2+} undergoes redistribution into the mitochondria on homogenisation should be indicated by a smaller Ca^{2+} content of organelles isolated in the presence of La^{3+} . Table 1 shows that slices homogenised with 1 mM La^{3+} yielded mitochondria containing 25% less Ca^{2+} than those homogenised in its absence. In a further series of experiments, La^{3+} at concentrations of 0.1, 0.5 and 2.0 mM reduced the mitochondrial Ca^{2+} from a control value of 47 ± 5 mmol per kg protein to 42 ± 3 , 42 ± 2 and 38 ± 2 mmol per kg protein, respectively—that is, a fall of 20% at 2 mM La^{3+} . We concluded that the maximal effect of La^{3+} was at 1 mM, and that 20–25% of the Ca^{2+} of mitochondria isolated in the absence of La^{3+} was derived from extramitochondrial sources on homogenisation. La can precipitate tissue proteins³ and when the ratio of La^{3+} to slice tissue in the homogeniser was $24 \mu\text{mol per g wet weight}$ we found a

reduction of 50% in the recovery of mitochondrial protein. The protein yield, however, was not reduced when the ratio was 8–12 $\mu\text{mol La}^{3+}$ per g wet weight, and this range was used in all the experiments we describe here.

The contribution of mitochondria to the total Ca^{2+} content of liver slices incubated in a Tris-buffered Ringer solution containing 1.3 mM Ca^{2+} was next determined in various conditions, subsequent homogenisation being carried out in the presence of 1 mM La^{3+} . The slices underwent a threefold increase in Ca^{2+} content at 1°C , and much of the extra Ca^{2+} was extruded during subsequent incubation at 38°C (Table 2). The mitochondrial Ca^{2+} changed in parallel with the total tissue content, showing a sixfold increase at 1°C and a subsequent recovery towards low Ca^{2+} levels at 38°C , which was even more complete than that of the whole slice. Whereas the mitochondria were calculated to contribute 35% of the total Ca^{2+} of the slices at 1°C , they only contributed 17% after recovery (Table 2). Preliminary experiments in which La^{3+} had not been used during homogenisation, showed qualitatively similar results whether slices were incubated in Tris-buffered or phosphate-buffered Ringer solutions⁴. In a few experiments the 'fluffy layer' was isolated and analysed separately; it was found to undergo qualitatively similar changes but with a Ca^{2+} content which was about 30% less, per unit protein, than the mitochondrial pellet proper. In contrast to the parallel changes of whole-slice and mitochondrial Ca^{2+} just described, the partial (35%) inhibition by cyanide of Ca^{2+} extrusion from the slices was not reflected in any inhibition of the loss of Ca^{2+} from the mitochondria (Table 2).

Mitochondrial K^+ participated in the overall loss of this

Table 2 Ca^{2+} and K^+ contents of liver slices and of mitochondria isolated from them after incubation in varying conditions

	Fresh tissue	90 min at 1°C	90 min at 1°C followed by 30 min at 38°C	
			Control	Cyanide (2 mM)
Ca^{2+}				
Intact slices				
(mmol per kg slice dry weight)	4.4 ± 0.8	12.8 ± 1.5	8.4 ± 0.8	10.0 ± 1.7
Mitochondria				
(mmol per kg protein)	7.3 ± 0.6	44.1 ± 3.7	11.9 ± 0.9	11.8 ± 2.5
(mmol per kg slice dry weight)	1.1	4.5	1.4	1.4
K^+				
Intact slices				
(mmol per kg slice dry weight)	317 ± 20	103 ± 6	173 ± 11	88 ± 5
Mitochondria				
(mmol per kg protein)	147 ± 3	91 ± 2	84 ± 4	37 ± 3
(mmol per kg slice dry weight)	21	9	10	4

Slices were incubated in Tris-buffered Ringer solution (see Table 1) as indicated, the vessels being gassed with O_2 before incubation at 38°C . Samples of the slices were taken for analysis after incubation; the remainder were homogenised in the presence of 1 mM La^{3+} and their mitochondria isolated together with the 'fluffy layer'. The quantities of intramitochondrial ions are expressed per kg mitochondrial protein and per kg tissue dry weight, the latter being calculated from the recovery of mitochondrial protein per unit of slice dry weight. The results shown are mean $\pm$ s.e. of the mean from three experiments, each carried out in triplicate.

ion from slices incubated at 1 °C and the further loss at 38 °C in the presence of cyanide (Table 2). Active aerobic metabolism at 38 °C, however, did not lead to restoration of mitochondrial K⁺ levels, although supporting reaccumulation of total slice K⁺. Mitochondria accounted for rather less than 10% of the total K⁺ content of the slices.

We have shown that addition of La³⁺ to the homogenisation medium inhibits the accumulation of both endogenous and exogenous Ca²⁺ by liver mitochondria to an extent sufficient to permit determination of the quantities of intra- and extramitochondrial Ca²⁺ that existed in the intact cell. The results of Table 2 provide direct experimental evidence that the Ca²⁺-accumulating system of mitochondria functions in the intact liver cell and that the accumulation is reversible, thus complementing the findings of Rose and Loewenstein¹⁵ who used intracellular aequorin as an indicator of cytosolic Ca²⁺ in intact cells of a salivary gland. Accessibility of Ca²⁺ to, and its retention by, the mitochondria seems to be controlled to a large extent by an opposing energy-dependent mechanism which maintains a low total cellular Ca²⁺. This calcium-extruding system may be situated either in the plasma membrane⁷ or in a region of the endoplasmic reticulum communicating with the extracellular fluids⁹. Inhibition of the extruding system at 1 °C permits entry of Ca²⁺ into the cytosol from the exterior, and from there to be accumulated by the mitochondrial system which remains active at 0 °C (ref. 4). Restoration of aerobic metabolism at 38 °C leads to withdrawal of Ca²⁺ from the mitochondria due, presumably, to a greater affinity of the extruding system at the cell surface. The fall of mitochondrial Ca²⁺ at 38 °C in the presence of cyanide, in spite of partial inhibition of extrusion from the cell, is probably due to a more complete inhibition of the mitochondrial system (which is totally dependent on respiration) than of the system at the cell membrane, since the latter would be able to utilise ATP derived from the continuing glycolysis. These considerations suggest that mitochondria may be able to regulate Ca²⁺-dependent aspects of cell metabolism as postulated by a number of workers (for example, refs 1, 2, 16 and 17), but that this regulatory effect would itself be modulated by the activity of the calcium-extruding system at the cell boundaries.

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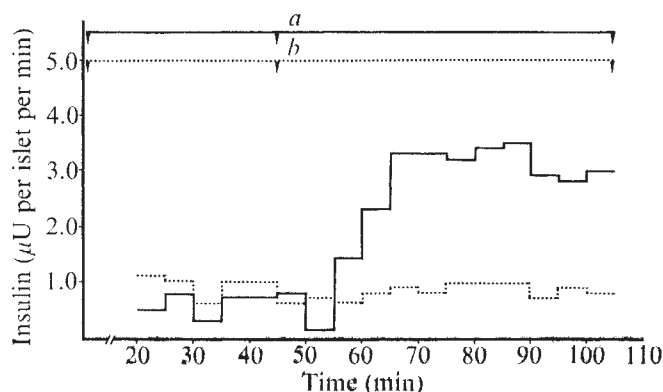
Increasing intracellular sodium triggers calcium release from bound pools

AN increase in the free ionised cytosolic Ca²⁺ concentration is generally regarded as the critical event necessary for the induction of exocytotic release of neurotransmitters and hormones from excitable cells. Stimulation of such cells is thought to result in an alteration of the membrane permeability to Ca²⁺ with a subsequent flow of Ca²⁺ ions down their gradient into the cytosol from the extracellular space¹. In addition, release of Ca²⁺ from intracellular compartments—notably the mitochondria—mediated by a rise in intracellular cyclic AMP may also, in some systems, result in an increase in the cytosolic Ca²⁺ concentration and thus to exocytotic release². Recent evidence suggests that small increases in Na⁺ concentration may release Ca²⁺ from mitochondria isolated from cardiac muscle cells³. Conversely, the uptake of Ca²⁺ into mitochondria of squid axoplasm is reduced by elevated sodium⁴. Since increases in the internal Na⁺ concentration occur during stimulation of many excitable cells, it is feasible that Na⁺ could act as a physiological releaser of Ca²⁺ from intracellular stores. We have obtained evidence for this possibility from studies designed to examine the role of Na⁺ in the calcium-dependent exocytotic release of insulin from the pancreatic β cell.

Pancreatic islets of Langerhans were isolated from 8-week-old male albino rats (200-250 g) and placed in a perfusion system which permitted the temporal monitoring of insulin release from their β cells⁵. Using this system, the effects of veratridine, an alkaloid well known for its ability to increase Na⁺ permeability in nerve cells⁶, were examined to test the possible role of Na⁺ in Ca²⁺-dependent insulin release. Our studies show that veratridine leads to a large release of insulin which is totally blocked by tetrodotoxin (TTX), a specific inhibitor of sodium channels in excitable membranes.

A typical experiment is presented in Fig. 1. Veratridine also stimulates release of neurotransmitters from synaptosomes⁷ and noradrenaline from the hypogastric nerve⁸,

Fig. 1 Islets of Langerhans were isolated by the collagenase technique¹³. An exact number of islets (80-100) were placed in each of two perfusion chambers and perfused with Krebs-Ringer-bicarbonate (KRB) of the following ionic composition: Na⁺ 139 mM; K⁺ 5 mM; Ca²⁺ 2.5 mM; Mg²⁺ 1 mM; Cl⁻ 127 mM; HCO₃⁻ 24 mM. This was supplemented with bovine serum albumin (5 g l⁻¹) and 5.6 mM D-glucose, and maintained at 37 °C and pH 7.40 by bubbling constantly with 95% O₂ and 5% CO₂. Islets in both chambers were exposed to this solution for 45 min, after which one chamber was perfused with KRB containing 100 μ M veratridine (a) and the other with KRB containing 100 μ M veratridine and 3 μ M TTX (b). The perfusate was collected at timed intervals and the insulin content determined by radioimmunoassay¹⁴. Veratridine caused a fourfold increase in insulin release that was completely inhibited by TTX.



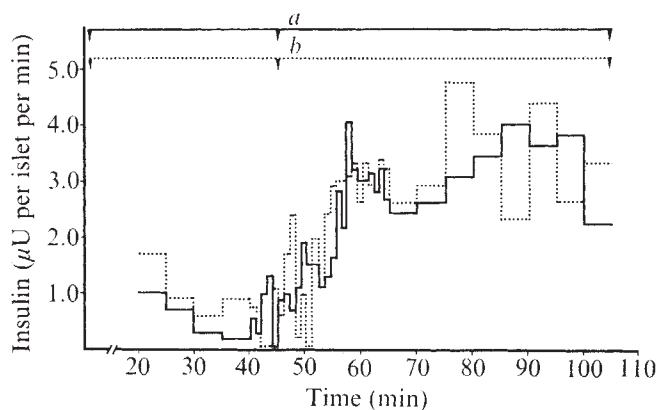


Fig. 2 Same procedure as in Fig. 1. After being perfused for 45 min with KRB alone, islets in one chamber were perfused with KRB containing 100 μ M veratridine (a) and the others with Ca^{2+} -free KRB supplemented with 100 μ M veratridine and 1 mM EGTA to chelate any remaining extracellular Ca^{2+} (b). Veratridine caused a fourfold increase in insulin release that was independent of extracellular Ca^{2+} .

release in both systems being blocked with TTX. Our original interpretation was that veratridine increased the intracellular Na^+ concentration by the maintained opening of Na^+ channels in the β -cell membrane, thus depolarising the membrane and opening voltage-sensitive Ca^{2+} channels. Ca^{2+} may, in addition, have entered the cell by inward transport in exchange for outwardly transported Na^+ , by counter-ion transport mechanisms similar to those identified in the squid axon⁹.

Repetition of our experiments, however, in Ca^{2+} -free buffer, containing 1 mM EGTA to chelate any remaining extracellular Ca^{2+} , resulted in the same amount of veratridine-stimulated insulin release as when Ca^{2+} was present (Fig. 2). Since insulin release is always thought of as being strictly Ca^{2+} dependent¹⁰, we suggest that increasing intracellular Na^+ concentration causes release of Ca^{2+} from intracellular compartments. Another method of increasing the intracellular Na^+ concentration is to poison the Na^+ pump with ouabain¹¹. We have demonstrated that ouabain also causes insulin release in the absence of extracellular Ca^{2+} . Ouabain-induced release of acetylcholine from neuromuscular endplates also occurs in the absence of Ca^{2+} (ref. 12). Thus insulin release in the absence of Ca^{2+} is not a phenomenon specific to veratridine, but one related to the elevation of intracellular Na^+ . In the light of these findings consideration must be given to the possibility that Na^+ , by releasing Ca^{2+} from intracellular pools, is acting as a 'second messenger' in exocytotic release processes.

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Interganglionic variation in cell body location of snail neurones does not affect synaptic connections or central axonal projections

RECENT accounts of invertebrate nervous systems have emphasised constancy in the occurrence and electrical properties of large identifiable neurones^{1–5}. That constancy, however, has limits, and some giant neurones show variation in peripheral axonal projections¹ and dendritic morphology^{6–8}. Smaller neurones increase in number during post-embryonic growth in several groups of invertebrates⁹. During electrophysiological mapping experiments on the brain of the pulmonate pond snail, *Lymnaea stagnalis* (L.), we have found two giant neurones whose positions varied considerably between ganglia, although the variation in cell body position was not associated with any variations in the synaptic connections of the neurones with each other or in identifiable synaptic inputs from other neurones, or with variations in the final projection areas of the principal axons within the brain. Some variation in the detail of axon branching was found to be correlated with cell body location for one peripheral projection.

Intracellular recordings were made from the cell bodies of neurones in the isolated brain maintained in snail blood, using standard electrophysiological techniques. Glass micro-electrodes were filled with 2 M K_2SO_4 or 6% Procion Yellow M-4RS. Procion dye was injected intracellularly by iontophoresis¹⁰ to determine axonal morphology of one or two cells in a particular brain. Brains were processed histologically in the conventional way. Axonal morphology was reconstructed from superimposed camera lucida drawings of serial sections. No detailed analysis of dendritic morphology was made.

The two neurones which show interganglionic variation in cell body location were called LP2 and LP3 and could not be separated from one another on the basis of their electrical and morphological characteristics. Their bright orange cell bodies were 100–150 μ m in diameter depending on snail weight, and the combination of size, positions, electrical properties and morphology of LP2 and LP3 made them uniquely identifiable in the brain of *Lymnaea*.

The somata of LP2, LP3 occurred as a pair in the visceral ganglion (49 snails) or left parietal ganglion (38 snails) or one in each of these ganglia (23 snails). Less frequently they occurred as a pair in the left pleural ganglion (3 snails) or one in the left pleural ganglion and one in the left parietal ganglion (2 snails) or one in the left pleural ganglion and one in the visceral ganglion (1 snail). The location of cell bodies within these three ganglia was limited to small areas of the ventral surface with typical positions shown in Fig. 2.

The alternative positions of LP2, LP3 cell bodies did not affect the connections between the two cells or connections with other interneurons. Resting membrane potentials were within the range -45 to -60 mV, and the cells were invariably connected by an electrotonic synapse, even when the cell bodies were in different ganglia (Fig. 1a and b). The junction between the cells was non-rectifying, and the coupling ratio for square pulses was in the range 0.05–0.15.

Spontaneous chemically mediated inhibitory postsynaptic potentials (i.p.s.p.s) were recorded in LP2 and LP3 originating from unidentified interneurons within the brain (Fig. 1, c–e). i.p.s.p.s were found to be remarkably synchronous and often of similar amplitude in pairs of LP2 and LP3 (Fig. 1 c–e). This shared input was seen wherever the cell somata were located. In many preparations two identifiable unitary i.p.s.p.s were seen (Fig. 1 c–e, i.p.s.p.s 1 and 2).

Procion dye injections of LP2 and LP3 showed that they

were monopolar cells with two principal axonal routes through the brain. The largest axon projected from the cell body anteriorly and then ventrally to the left pedal ganglion ("pedal axon", Figs 1f and 2) while a second finer branch, originating from the larger close to the cell body, projected posteriorly to the visceral ganglion ("visceral axon", Figs 1f and 2). When both neurones occurred in the same ganglion their axonal pathways through the central neuropile

were extremely similar (Fig. 1f), although there were sometimes differences in the peripheral nerves into which they projected. When the cell bodies were in different ganglia their proximal axons met in the brain neuropile and then shared common pathways to the same final ganglion. To reach the same destination irrespective of cell body location required different lengths of pedal and visceral axons (Fig. 2).

One peripheral nerve axon branch of LP2, LP3 could originate from either principal axon depending on the ganglion in which the cell body was located. This was the constantly occurring left perietal nerve branch which emerged from the principal axon at a point where it passed close to the left perietal nerve root (in 37 out of 38 injected neurones). When the cell body was in the left pleural ganglion (3 cells injected) the branch originated from the visceral axon (Fig. 2a) but when the cell body was in the visceral ganglion (11 cells injected) it branched from the pedal axon (Fig. 2c). In cell bodies located in the left parietal ganglion it mostly branched from the pedal axon (in 21 injected cells) but occasionally from the visceral axon (in 3 injected cells) (Figs 1f and 2b). Thus the axon branch to the left perietal nerve is formed during development from whichever principal axon passes through the central neuropile of the left parietal ganglion.

Some axon branches to peripheral nerves were variable

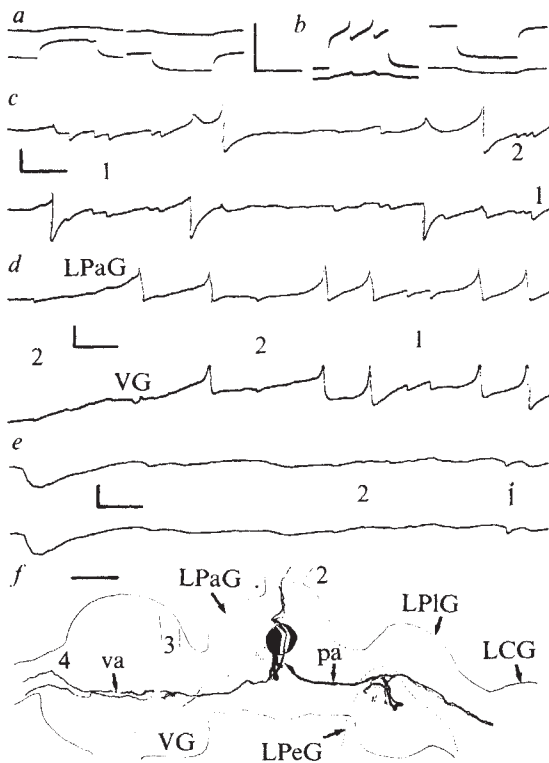


Fig. 1 Electrical properties and morphology of identified *Lymnaea* neurones, LP2 and LP3. *a*, Non-rectifying electrotonic synapse between a pair of neurones whose cell bodies were in the visceral ganglion. Two electrodes were placed in one cell body (bottom traces), one used for recording and the other for current injection, and a third electrode in the second cell body to record the postsynaptic response (top traces). 1-s square current pulse, 2×10^{-9} A, both polarities. Coupling ratio is 0.12 for depolarising (left-hand trace) and hyperpolarising currents (right-hand trace). *b*, Same but for neurones with cell bodies in the visceral ganglion (top traces) and left parietal ganglion (bottom traces). 1×10^{-9} A square current pulse, 1-s duration injected into the visceral neurone by the recording electrode. Coupling ratio for left and right traces, 0.07. Scale bars refer to *a* and *b*. *a*, horizontal, 0.5 s; vertical, 20 mV (top traces), 40 mV (bottom traces). *b*, horizontal, 0.5 s; vertical, 80 mV (top traces), 40 mV (bottom traces). *c-e*, Spontaneous i.p.s.p.s recorded in paired recordings when the cell bodies were located both in the visceral ganglion (*c*) one in the left parietal ganglion (LPaG) one in the visceral ganglion (VG) (*d*) and both in the left parietal ganglion (*e*). Synaptic inputs to pairs of cells are synchronous and of approximately equal amplitude. Identified unitary i.p.s.p.s are marked 1 and 2 in records (*c-e*). In *d* continuous small i.p.s.p.s of a third type are present throughout the records and they probably form the principal component of the compound i.p.s.p.s seen at the beginning of the trace in (*e*). *f*, Reconstruction of a pair of Procion-injected neurones with cell bodies on the ventral surface of left parietal ganglion (LPaG). In the animal the left cerebral ganglion (LCG, the most anterior ganglion in the snail) is in the same horizontal plane as the left pleural ganglion (LPIG) the left parietal ganglion and the visceral ganglion (VG) whereas the left pedal ganglion (LPeG) is ventral to the others (dashed outline). Note the similar axonal geometry of the two cells. The visceral axon (va) projects to the anal nerve (4) but not to the cutaneous pallial nerve (3) in both cells. The pedal axons (pa) could not be traced to the pedal nerves although they penetrate the pedal ganglion neuropile. The left parietal nerve branch (2) arises from the visceral axon in one neurone (shaded) and the pedal axon in the other (unshaded). Scale bar, 200 μ m.

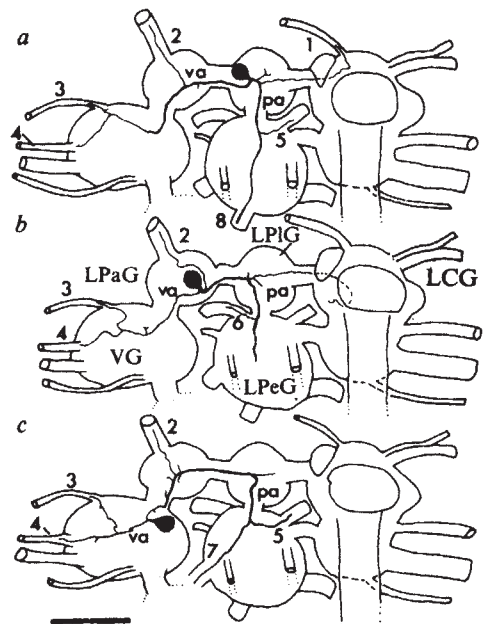


Fig. 2 Reconstructions of Procion Yellow injected LP2, LP3 drawn into standard diagrams of the left side of the *Lymnaea* brain. Cell bodies located *a*, in the left pleural ganglion (LPIG); *b*, in the left parietal ganglion (LPaG) and *c*, in the visceral ganglion (VG) on the ventral surfaces are shown for comparison. For simplicity only one of the LP2, LP3 neurones is included in each diagram. If the second cell occurred in the same ganglion as the one shown in *a-c* then its cell body was located next to the first (see Fig. 1f) and its central axonal projections were similar. The left cerebral ganglion (LCG) is most anterior in the brain and the left pedal ganglion (LPeG) most ventral. The cell body location determines the lengths of the visceral axon (va) and the pedal axon (pa). In the sequence *a-c* the visceral axon gets shorter and the pedal axon longer. The left parietal nerve branch (2) originates from the visceral axon in (*a*) and the pedal axon in (*b*) and (*c*). All three cells possess a peripheral axonal branch to the anal nerve (3) and the cutaneous pallial nerve (4). The axon branches to the pedal nerves are variable (see text for discussion). Only in the case of the cell located in the left pleural ganglion (*a*) was it possible to trace the cerebral ganglion axon branch to the nuchal nerve (1). Labelling of pedal ganglion nerve roots; 5, superior cervical nerve; 6, inferior cervical nerve; 7, inferior pedal nerve; 8, medial pedal nerve. Scale bar, 500 μ m.

in occurrence. The apparent variability of pedal nerve projections seen in Fig. 2 may partly be due to problems of tracing distal nerve processes which have little dye in them, but in the visceral ganglion (Figs 1f and 2) axon branches were easy to map and some variation in branching pattern is certain. This was not obviously related to the location of the cell body. Thus in some snails the visceral axon projected to the anal nerve (this occurred in four cell bodies located in the visceral ganglion, and four cell bodies located in the left parietal ganglion), in others it projected to the cutaneous pallial nerve (in three cell bodies located in the visceral ganglion, one cell body located in the left parietal ganglion and one cell body located in the left pleural ganglion) or it projected to both these nerves (in three cell bodies located in the visceral ganglion and in three cell bodies located in the left parietal ganglion).

The constant presence of certain synaptic connections which LP2 and LP3 make shows that the position of cell bodies need not be constant for normal neural connections to be made. It seems more important to specify axonal projections rather than cell body position and this is suggested by the present study as well as that of Selverston and Mulloney¹¹ where cells from the lobster stomatogastric ganglia varied considerably in cell body location but had constant neuropile axon structure.

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Absence of a plateau in length–tension relationship of rabbit papillary muscle when internal shortening is prevented

IN the length–tension relationship of the isolated papillary muscle there is a region in which increased muscle length is associated with an increased force of contraction. Several problems make it difficult to determine the physiological basis for this relationship. Thick and thin filament lengths and sarcomere lengths have not been determined with the same accuracy in heart muscle as in skeletal, though there is evidence that these lengths are nearly the same in both muscles^{1–3}. Papillary muscle cannot normally be tetanised or made to contract at low temperatures, and there is reason to believe that it is not fully activated by a single stimulus in the usual bathing medium^{4,5}. In at least some papillary muscles there is considerable internal shortening during a twitch contraction^{6,7}. Such length changes during contraction could change the amount of force developed by affecting the amount of thick and thin filament overlap and by producing shortening deactivation^{8,9}. We have developed a photoelectronic servo apparatus similar to that



Fig. 1 A low power view of a typical rabbit papillary muscle preparation. The tapered tendon end and cut end are knotted to stainless steel wires with 10-0 nylon monofilament sutures. As a reference length, the diameter of the wires was 200 μ m. The muscle was stretched to L_m . Its diameter in the uniform central region was about 500 μ m, while its total length (knot-to-knot) was about 3.7 mm. The markers were about 1 mm long by 0.25 mm wide and were made from gold foil (2.5 μ m thick), folded over along each long side, so that total marker thickness was 5 μ m. Markers were glued to the surface of the papillary muscle with an epoxy adhesive able to cure underwater. The length between the markers (edge-to-edge) was about 1.7 mm. The dark streaks and spots visible in the muscle are collections of opaque 15- μ m diameter microspheres; their presence does not influence muscle function.

developed by Gordon *et al.* (see refs 10 and 11 for details of operation) for use with papillary muscles to hold constant the length of a segment of preparation during contraction.

Small diameter papillary muscles were obtained from the right ventricles of anaesthetised rabbits (about 0.5 kg). When these muscles were stretched to near L_m , the length at which peak developed tension is maximum, the diameter measured in the mid-region was about 500 μ m, and striation patterns could be observed and recorded as described before⁶. The bathing medium was a modified Krebs–Ringer solution bubbled with a 95% O₂–5% CO₂ mixture. The solution contained (mM): NaCl, 117; NaHCO₃, 1.4; KCl, 4.2; CaCl₂·2H₂O, 2.5; MgSO₄·7H₂O, 1.2; NaH₂PO₄, 1.2; HEPES buffer, 7.4, and glucose, 5.6.

When a suitable muscle was obtained, it was tied to wires⁶ (Fig. 1), which were connected to a servo motor and force transducer. The muscle was stretched slowly to just beyond L_m while being stimulated repeatedly. The experimental chamber was then drained of solution and gold markers (Fig. 1) were rapidly attached (within several minutes) to a central, uniform segment of the papillary muscle. The chamber was then refilled with solution and stimulation resumed. Comparison of length–tension relationships before and after marker attachment revealed no significant differences, and the muscles contracted vigorously for several hours after marker application. Microspheres which had been injected into the beating heart and subsequently lodged in the papillary muscle capillary network, were examined microscopically during fixed-end contractions to ensure that the markers moved synchronously with other elements distributed throughout the muscle cross section. Such synchrony was taken to indicate that during servo control, internal length does not change when the separation between the markers located on the muscle surface is kept constant.

The fixed-end length–tension relationship obtained from a papillary muscle after markers had been attached, but without servo control, is shown in Fig. 2. Peak twitch force declines to nearly zero when total muscle length is decreased by about 25% of L_m , and there is only a very narrow (about 1% of L_m) plateau present over which peak developed force remains near maximal. The steeply rising resting tension made it impossible to stretch the muscle more than a small amount beyond L_m . Servo control was used at each of the lengths indicated by arrows in Fig. 2 to prevent changes in marker separation. In each case, control was increased progressively to the point where only a small change in marker separation occurred during a twitch. Further increases in servo gain were avoided, since at higher gains the servo system became marginally stable. Only the record obtained at the longest indicated length is

shown in Fig. 3, the others being similar in form. As the change in marker separation is progressively reduced, there are significant increases in the rate of rise of force and twitch peak amplitude. There is also an increase in the half time for twitch tension decay and a tendency for the twitch peak to occur slightly earlier.

Data obtained from each of the five records of the kind just described are plotted in Fig. 3, where it can be seen that the relative length error decreases monotonically as servo gain is increased. At maximum gain, the relative length error is less than 1%, and is even smaller at the two longest lengths, where large resting tensions were present. The extrapolated values give the best estimates of the force which would be present if marker separation had been held absolutely constant during a twitch contraction. The extrapolated values for the forces do not differ much from the values observed with minimum relative length error. These results provide a reasonable basis for belief that the small residual length error present did not significantly affect the force responses, so that the active tension developed indicates unambiguously force generation by the contractile component.

It is now possible to plot peak developed force against the length between the markers at maximal servo system gain (Fig. 4). The largest relative marker separation corresponds to a muscle length very near to L_m . The average sarcomere length corresponding to the greatest length between the markers was found by photographing the striation pattern⁶ in the resting muscle. A mean value of

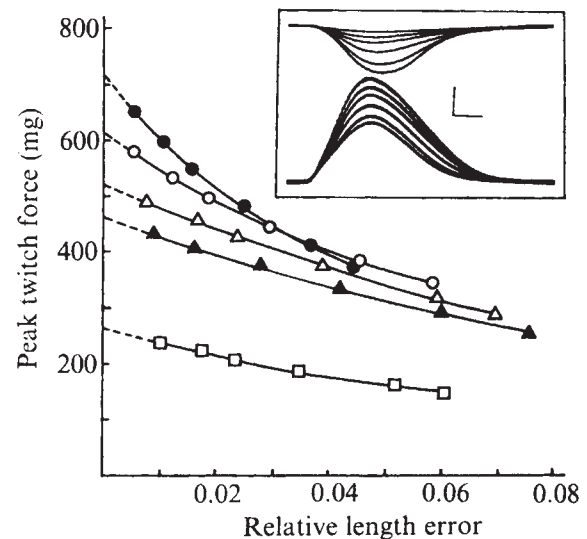


Fig. 3 Peak twitch force plotted against relative length error. Data were obtained at each of the positions indicated by arrows in Fig. 2 from records of the kind shown in the inset. In each case, as servo gain increased progressively, the maximum decrease in length produced by each twitch was obtained with the corresponding twitch amplitude. Decreases in length were expressed relative to the initial, resting length between the markers, so that a relative length error was obtained. Each set of data appears in sequence from top to bottom in correspondence with the arrows in Fig. 2. As servo gain was increased, the amount of relative length error decreased, that is, a nearly perfect isometric clamp was attained. In each case, the points are well fitted by a smooth curve. The extrapolations of these curves (dashed line segments) on to the force axis give good estimates of the true isometric forces obtainable if marker separation were held constant during a twitch. In sequence from top to bottom, the initial, resting marker separations were (mm): 1.60, 1.56, 1.52, 1.48 and 1.37. Inset: a sequence of length and force records showing the effects of increasing amounts of servo control applied to the papillary muscle from which the record shown in Fig. 2 was obtained. The upper series of traces are length signals from the photoelectric spot follower. These indicate changes in marker separation, where length decreases are indicated by a downward deflection. A corresponding series of twitches is shown below. The smallest twitch is associated with the largest deflection in the length signal records. Between each twitch, the gain of the servo system was increased. This caused the end of the muscle attached to the servo motor to be driven so as to diminish progressively the amount of change in marker separation during a twitch. Finally, servo gain was increased to the point at which, during a twitch, there was a just perceptible deflection in the length signal trace. These records were obtained at the resting total muscle length indicated by the arrow in Fig. 2 closest to the active force peak. Similar sequences were obtained at the other four positions indicated by arrows in Fig. 2, but they are not shown here. At maximum gain, an error of 1 μm in the length between the markers causes an opposing force of about 73 mg at the end of the muscle attached to the servo motor. The vertical calibrating line indicates 44 μm or 182 mg, and the horizontal calibrating line indicates 200 ms. The largest twitch amplitude corresponds to a tension of about 8 g mm^{-2} .

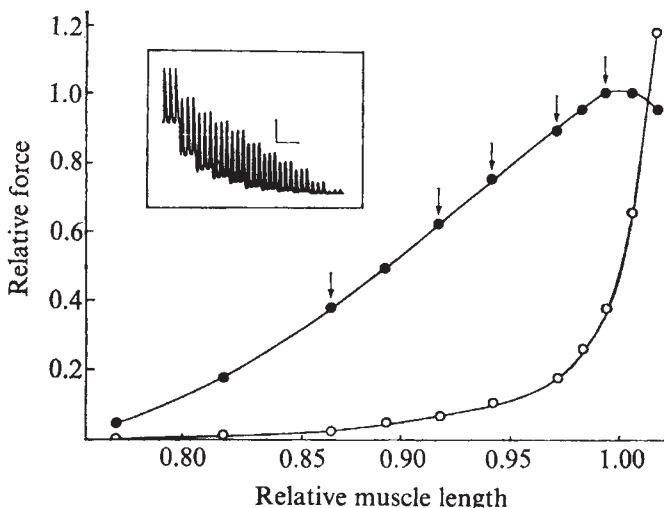


Fig. 2 A plot of peak developed (●) and resting (○) tension against muscle length for a rabbit papillary muscle. Data were obtained from the experimental record shown in the inset. Forces are expressed relative to the amplitude of the maximal peak developed twitches. Total muscle length (knot-to-knot) is expressed relative to L_m . Inset: length-tension relationship of a rabbit right ventricular papillary muscle. Markers had previously been attached as shown on a different muscle in Fig. 1. The muscle was first stretched slowly (not shown) until peak developed twitch tension passed a maximum and then declined slightly. After this, muscle length was decreased progressively allowing three twitches at each length. At the shortest length, resting tension was nearly zero. The amplitude of the twitches after the first and second decreases in length was nearly equal. Total muscle length (knot-to-knot) was 4.1 mm at the greatest length and 3.1 at the shortest length. The size of the length decreases in order were (μm): 50, 50, 50, 50, 100, 100, 100, 200 and 200. Rate of stimulation was 17 per min, temperature was 22 °C. Calibration lines indicate 182 mg (vertical) and 14.4 s (horizontal). At the length corresponding to that after the second length decrease, muscle cross-sectional area was about 0.09 mm^2 , based on microscopic determination of diameter in the central region between the markers. Maximal peak developed twitch tension was about 5 g mm^{-2} .

2.3 μm was obtained for the average sarcomere length at a marker separation corresponding to L_m in three muscles. In Fig. 4 the sarcomere length corresponding to a 15% decrease in marker separation 2.0 μm , was calculated by assuming a linear decrease of resting sarcomere length with marker separation, a good approximation in the sarcomere length range under consideration⁶. Figure 4 shows that there is a marked variation of twitch force within a range of average sarcomere lengths from about 2.0 to 2.3 μm , which contrasts with the results for frog skeletal muscle fibres in which isometric tetanic tension is essentially constant within this range¹¹.

The results presented here indicate that most of the ascending limb of the length-tension relationship of rabbit

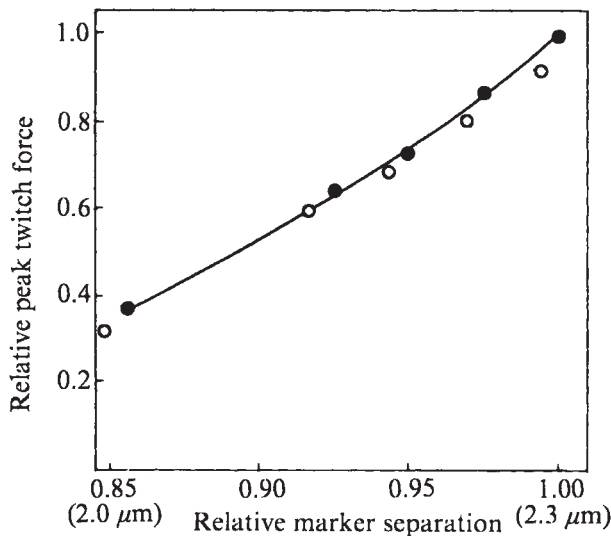


Fig. 4 Peak twitch force as a function of marker separation. Data were obtained from the graph shown in Fig. 3. All forces are expressed relative to the largest obtained by extrapolation, and all marker separations are expressed relative to the longest initial, resting value, 1.60 mm. ●, Plot of extrapolated force values against marker separation for the case of zero relative length error, that is the best estimates for perfectly isometric conditions. ○, Actual final values for forces and marker separations under maximum servo gain conditions. Note that a decrease in marker separation of only about 15% leads to a decrease in force of more than 60%. Average sarcomere lengths are given in parentheses. The value 2.3 μ m was obtained by microscopic examination of the resting muscle at marker separation 1.60 mm. The value 2.0 μ m was obtained by assuming a linear decrease of resting sarcomere length with marker separation.

papillary muscle cannot simply be explained on the basis of variation in the amount of thick and thin filament overlap, and, therefore, numbers of cross bridges available for interaction, or by changes in the amount of filament overlap and deactivation caused by internal shortening during contraction. Results obtained from skeletal muscle suggest that a variation in level of activation with muscle or sarcomere length is a significant factor¹²⁻¹⁴. This is, of course, not a new proposal¹⁵⁻¹⁸. Recent experiments with skinned muscle cells obtained from rat ventricles are interesting in this regard¹⁷. If these cells, which have had their sarcolemmas removed mechanically, are activated directly by high concentrations of calcium ion in the presence of a large Ca^{2+} -buffering capacity, the resulting sarcomere length-tension relationship resembles that obtained from living frog skeletal fibres¹¹. Steady tension varies by only about 10% in the range of sarcomere lengths from about 1.7 to 2.3 μ m, and it varies even less within the range 2.0 to 2.3 μ m. But when the skinned cells were made to contract phasically by inducing a Ca^{2+} -triggered transient release of Ca^{2+} from the sarcoplasmic reticulum (SR), tension decreased substantially as sarcomere length was reduced below 2.3 μ m. In the work with living papillary muscles reported here, tension decreased even more rapidly as sarcomere length was reduced below 2.3 μ m than in the phasic contractions of skinned heart cells¹⁷. This suggests that in the living muscle the mechanism responsible for regulating the amount of activating calcium released in a twitch is not necessarily the same as that which governs the calcium-triggered release of calcium from the SR of skinned cells.

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Electrophysiological measurements of spectral sensitivity of central visual cells in eye of blowfly

IN 1913 von Frisch was able to show that bees can discriminate between different colours. He thus refuted the then prevailing opinion that only certain vertebrates have colour vision. Since then insect colour vision has become a significant subject of research, the more so in view of the fact that using insect visual cells the transformation of light into neural excitation can be readily examined. In recent years numerous electrophysiological, microspectrometric and behavioural experiments have been carried out to determine the spectral sensitivity of visual cells not only of the bee but also of the fly. In the case of the blowfly *Calliphora erythrocephala*, each compound eye is made up of approximately 3,000 ommatidia. Under the lens of each ommatidium there are eight visual cells, and these can be divided into two groups according to their shape. Six of them (numbered R_{1-6}) have short axons which end in the first optic ganglion (lamina ganglionaris). The remaining two visual cells (R_{7+8} , also called 'central visual cells' because of the position they occupy in the ommatidium) are characterised by their long axons, which stretch from the retina through the lamina ganglionaris down to the second optic ganglion (medulla).

Measurements by electrophysiological methods have given rise to double-peaked sensitivity curves¹⁻³, all having in common a maximum in the ultraviolet range at 350 nm. The other peaks occurred at different wavelengths in the visible range of the spectrum. According to the positions of these maxima Burkhardt¹ was able to divide the sensitivity curves into three categories which he ascribed to three colour receptors (blue receptor: $\lambda_{max} = 475$ nm, green receptor: $\lambda_{max} = 493$ nm, yellow-green receptor: $\lambda_{max} = 515$ nm). The frequency of occurrence found for the sensitivity curves in each category led him to ascribe the green receptor to visual cells R_{1-6} ; and the blue and yellow-green receptor to central visual cells R_{7+8} . Microspectrometric measurements⁴ confirmed this assumption in the case of visual cells R_{1-6} . For the visual cells R_{7+8} together (these are positioned in line with one another and share one rhabdomere) absorption spectra were found which, in

addition to an ultraviolet maximum, had a maximum at 475 nm. Electrophysiological measurements with simultaneous histological localisation of the cells in question showed that the spectral sensitivity with a maximum at 490 nm did in fact come from the visual cells R_{1-6} . Such a definite proof has not been possible for visual cells R_{7+8} taken separately.

To determine the spectral sensitivity of the central visual cells R_{7+8} , intracellular potentials were recorded from the first optic chiasma. Since the axons of the visual cells R_{1-6} already terminate in the lamina we could be sure that whenever depolarising potentials with shapes typical for generator potentials from a retinula cell occurred, the recordings from the chiasma came either from the seventh or eighth visual cell. The measurements were carried out on the dark-adapted eye of male blowflies *Calliphora erythrocephala* Meig. with normal eye pigmentation. Many attempts were made but only in two cases were the recordings from the central visual cells stable enough to enable complete measurement of the intensity-response curve and of the spectral efficiency. Using intracellular injection of the fluorescent dye Procion Yellow and subsequent histological examination, the exact recording site in the chiasma could be located. In one case only a part of a central visual cell became stained and therefore it was not possible to distinguish exactly between R_7 and R_8 .

Figure 1 shows the spectral efficiency curve of a central visual cell (response elicited by light stimuli with the same quantum level at all wavelengths) and its spectral sensitivity curve

Fig. 1 Spectral efficiency (a) and spectral sensitivity (b) of a central visual cell from eye of *Calliphora*. Single-peaked ultraviolet receptors of this kind were previously unknown in the blowfly eye.

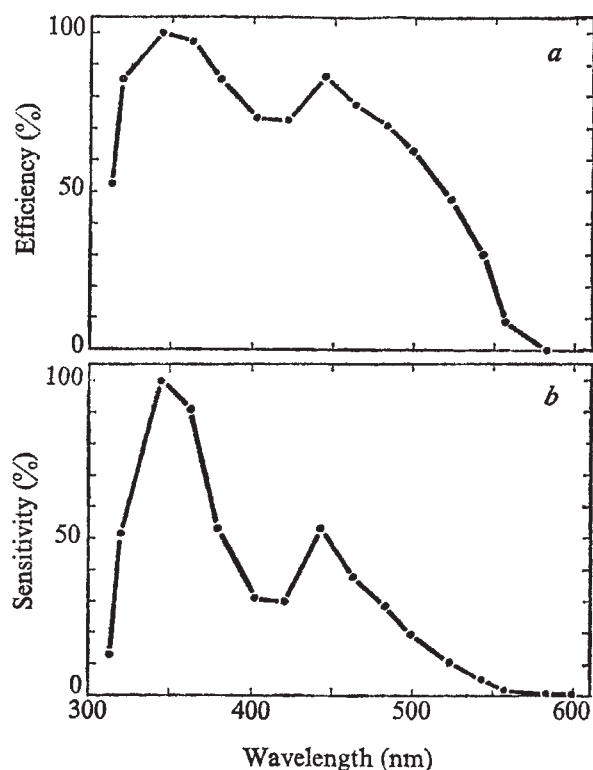
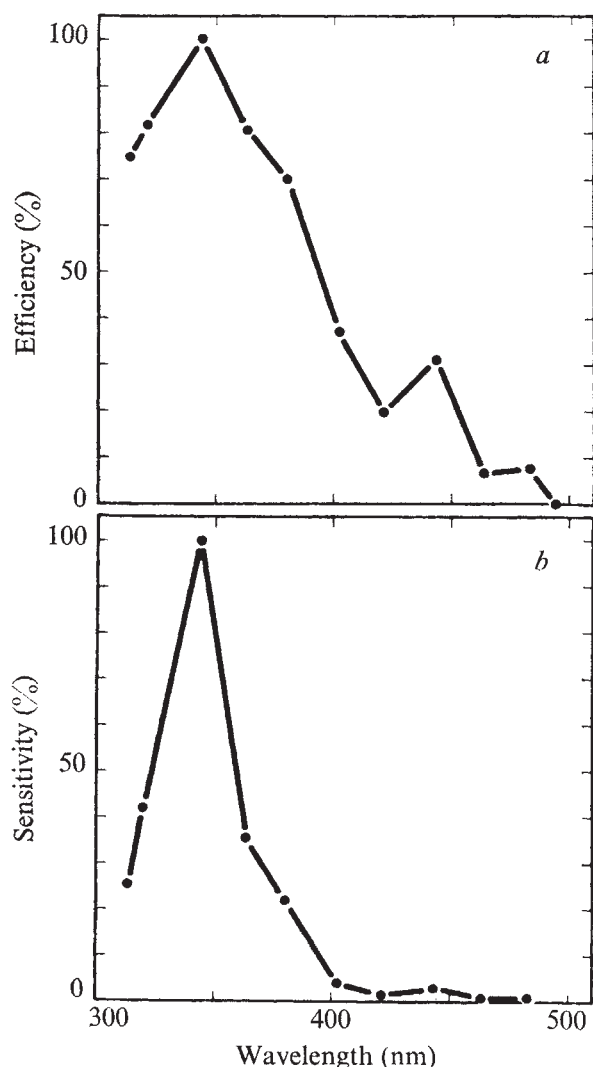


Fig. 2 Spectral efficiency (a) and spectral sensitivity (b) of another central visual cell which in addition to the ultraviolet maximum also shows a marked maximum at 443 nm in the blue range.

(reciprocal of quantum level eliciting the same response at all wavelengths). The spectral efficiency (Fig. 1a) of this cell has its maximum in the ultraviolet range at 344 nm, and drops sharply as the wavelength increases. At 443 nm it shows a small subsidiary shoulder and at 500 nm the spectral efficiency is almost zero. The spectral sensitivity curve (Fig. 1b) shows very clearly a single-peaked ultraviolet receptor. Here, the subsidiary shoulder in the blue range has declined to a level 10% below maximum sensitivity. Figure 2 shows the spectral efficiency and sensitivity curve of another central visual cell, differing in shape from that in Fig. 1. The efficiency curve (Fig. 2a) also shows a maximum in the ultraviolet range at 344 nm; but in this case the other maximum at 443 nm in the blue range was much more evident than in Fig. 1. At 500 nm the efficiency of this receptor is considerable and is still evident above 550 nm. The sensitivity curve (Fig. 2b) also shows two maxima the positions of which correspond with those in Fig. 1. The height of the blue maximum, however, is different. The axon of the cell mentioned in Fig. 2 was stained in the region between medulla and basement membrane. Faint staining could also be seen in the proximal region of the retina. Here, it was impossible to ascertain whether a seventh or eighth visual cell had been stained.

In electrophysiological experiments on the spectral sensitivity of visual cells in the eye of *Calliphora*, only double-peaked sensitivity curves have so far been found. Thus the occurrence of a single-peaked ultraviolet receptor is at first surprising⁵. The spectral sensitivity curve (Fig. 1b) suggests the existence of a special ultraviolet pigment in the rhabdomere of the seventh or eighth visual cell, such as has been isolated in *Ascalaphus*⁶. Ultraviolet receptors have also been found in other dipteran compound eyes⁷⁻⁹; thus they seem to be a characteristic of a number of dipterans. Also, the double-peaked sensitivity curves of the visual cells R_{1-6} in *Calliphora* can be ascribed to two separate pigments³. It is thus evident that the curves shown in Fig. 2 result from two visual pigments with maxima at 344 nm (ultraviolet pigment) and at 443 nm (blue pigment). The occurrence of such a blue pigment does not contradict earlier microspectrometric measurements on the rhabdomere of visual cells

R_{7+8} , for it is now well known that a stable metarhodopsin forms during these measurements, which shifts the maximum of the absorption curve towards longer wavelengths (H. Langer, personal communication).

We could not be certain which spectral sensitivity curve belonged to which visual cell; R_7 or R_8 . Experiments on *Drosophila*⁸ have shown, however, that the seventh visual cell is a single-peaked ultraviolet receptor whereas the eighth visual cell is a double-peaked ultraviolet-blue receptor. If one assumes that this also holds good for *Calliphora* the ultraviolet receptor (Fig. 1) would be the seventh visual cell and the ultraviolet-blue receptor (Fig. 2) the eighth visual cell. There remains, however, the possibility that not all the seventh visual cells and not all the eighth visual cells show uniform spectral sensitivities. Thus, the two different spectral sensitivity curves which we obtained might have come from two cells of the same type. If the ultraviolet pigment-blue pigment ratio varies from cell to cell this would be one explanation for the different maxima observed. This, for example, was the explanation suggested for the variation in height of maxima of spectral sensitivity curves with two or more peaks in *Eristalis*⁹. Microspectrometric measurements carried out on visual cells R_{7+8} of several dipterans do in fact show that neither of the rhabdomeres 7 and 8 constitutes an homogeneous population of receptors (K. Kirschfeld, personal communication). The question of which curve belongs to which cell type still remains to be answered, but our measurements show that, in addition to receptors with sensitivities found previously, there exist receptors with single-peaked ultraviolet sensitivities and with double-peaked ultraviolet-blue sensitivities. If one leaves out the possible shifting of the position of the blue maximum due to light-guide characteristics of the central rhabdomeres, one can conclude that the vision of the fly is based on at least three photopigments with maxima in the ultraviolet range (about 344 nm), blue range (about 443 nm) and green range (about 490 nm).

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Membrane noise in *Paramecium*

THE ciliated protozoan *Paramecium* provides an opportunity to study the electrophysiological properties of an excitable membrane in relation with simple locomotor behaviour. The *Paramecium* surface membrane controls the locomotor activity, in particular ciliary reversal (resulting in backward swimming), by ionic conductance mechanisms like those in other excitable cells^{1,2}. Depolarising stimuli (electrical, chemical or mechanical) cause a graded receptor potential due to a regenerative transient increase in calcium conductance and a delayed increase of potassium conductance. The resulting Ca^{2+} influx causes an increase in the intracellular Ca^{2+} concentration which initiates the reversal of the ciliary beating direction. Normal beating is resumed when the excess internal Ca^{2+} has been removed by metabolism-dependent processes.

The correlation between depolarisation and the orientation

of the cilia was first observed in relation with spontaneous fluctuations of the resting membrane potential^{3,4}. Spontaneous ciliary reversals seemed to coincide with voltage fluctuations in depolarising direction.

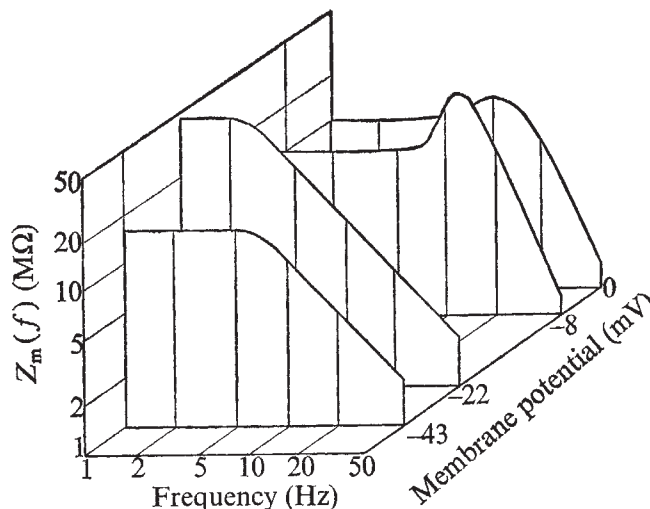
This report deals with the search for the physical origin of these fluctuations ("membrane noise") and with their relationship to spontaneous reversals. Membrane noise voltage (at constant current), although more easily measurable and less contaminated with electronic noise, may give less information about the underlying processes than noise current (at constant voltage), since the noise voltage is filtered by the frequency-dependent membrane impedance. The noise voltage is, however, expected to induce spontaneous ciliary reversals. We therefore measured both noise voltage and noise current and the impedance. We show that the membrane noise belongs to the so called $1/f$ type and that its intensity is related to the magnitude of the passive K^+ flux across the membrane. The noise voltage seems to be filtered by a resonance-like membrane impedance and, finally, we present evidence that $1/f$ noise can occasionally lead to spontaneous ciliary reversals.

Current clamp methods (using two intracellular micro-electrodes) similar to those of Naitoh and Eckert⁵ were used to record noise voltage from specimens of *Paramecium caudatum*. The noise voltage was perfectly correlated in both electrodes and independent of electrode positions, as expected from the isopotential nature of the *Paramecium* membrane⁶. Noise current was measured with standard voltage clamp techniques. The noise was tested for constancy and processed on an HP 3721 A correlator and Fourier transformed to obtain the spectral density function. The membrane impedance was obtained by cross correlation of injected white-noise current and membrane noise voltage response. In the "standard" bathing solution (4 mM KCl, 1 mM $CaCl_2$, 1 mM Tris, pH 7.2) the membrane noise was stationary and no spontaneous reversals were observed.

Figure 1 shows the membrane impedance modulus $|Z_m(f)|$ at different mean membrane potentials, measured from a specimen in the standard solution at 19 °C. At the resting potential (–22 mV) and on hyperpolarisation the membrane behaves as a simple RC circuit. On depolarisation, however, a resonance peak shows up at about 12 Hz. This resonance, which tends to diminish on stronger depolarisation, is reminiscent of the squid axon membrane impedance⁷ and accounts for the oscillatory character of the voltage response on a steady depolarising current stimulus⁸.

Figure 2a shows typical noise voltage spectra, $S_V(f)$, in which

Fig. 1 *Paramecium* membrane impedance modulus $|Z_m(f)|$ at various membrane potentials determined as (cross) power spectra of membrane voltage response to white noise current stimulus. R.m.s. value of injected noise was 5×10^{-11} A. In the standard bathing solution resting potential $V_r = -22$ mV and K^+ -equilibrium potential $E_K = -38$ mV at 19 °C.



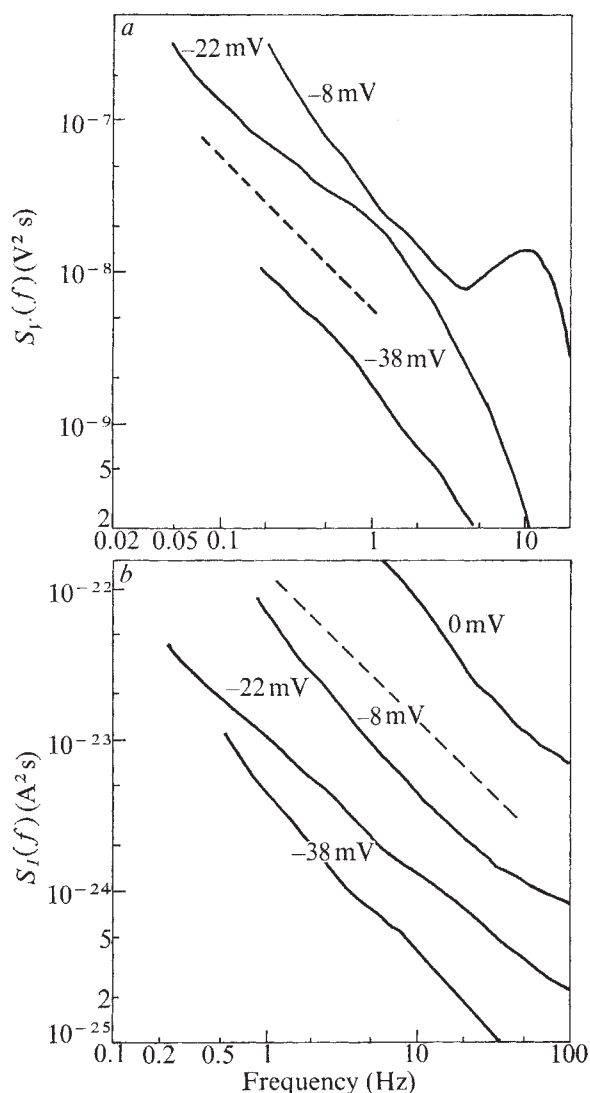


Fig. 2 Power spectra of stationary membrane noise at various membrane potentials. Standard solution at 19 °C. *a*, Noise voltage spectra, $S_V(f)$, measured under current clamp. Electronic noise negligible (10^{-11} V² s at 1 Hz). *b*, Noise current spectra $S_I(f)$, measured under voltage clamp. Electronic noise negligible up to about 50 Hz (10^{-25} A² s), but exceeding membrane noise above 100 Hz. Note the difference in frequency scale. Dashed lines represent slope -1 . Spectra are related according to $S_V(f) = S_I(f) |Z_m(f)|^2$.

the impedance filtering effect is clearly reflected. No significant changes in membrane noise spectra nor in other electrical parameters were observed when ciliary beat was inhibited by adding 1 mM NiCl₂ to the standard solution. This is consistent with the known independence of ciliary beat on electrical events².

Noise current spectra, $S_I(f)$, are shown in Fig. 2*b*. The experimental data satisfy the theoretical conversion formula⁸

$$S_V(f) = S_I(f) |Z_m(f)|^2$$

Noise current spectral densities decrease with increasing frequencies. On a log-log plot their shapes suggest a power law fit of the form

$$S_I(f) = Nf^{-\alpha}$$

where N is voltage dependent. α has a value close to 1.0 at rest and tends to increase on depolarisation. Values exceeding 1.5 were not found. This result places the membrane fluctuations of *Paramecium* in the $1/f$ -noise category.

This type of noise is also present in nerve fibres⁹ and in cultured neuroblastoma cells (W. H. Moolenaar, unpublished),

but unfortunately the physical basis of $1/f$ noise is not yet clear¹⁰. An empirical understanding of *Paramecium* membrane noise can, however, be obtained. Figure 3 shows the dependence of the noise intensity, N , on membrane potential V_m . A minimum value of N was found for V_m between -35 mV and -45 mV, that is the vicinity of the K⁺ equilibrium potential E_K ($E_K = -38$ mV in the standard solution¹¹).

Replacement of the standard solution by a high-potassium solution (18 mM KCl, 1 mM CaCl₂, 1 mM Tris, pH 7.2) roughly eliminates the potassium concentration gradient across the membrane, thereby reducing both E_K and V_m to about 0 mV. In this case membrane noise was no longer measurable since it vanished in electronic background noise, but hyper- or depolarisation raised the membrane noise intensity substantially once again. Although quantitative data on the I - V relationship for the separate ionic currents in *Paramecium* have not been reported as yet, the presented data strongly suggest a proportionality between membrane noise intensity and the magnitude of the passive K⁺ flux. We attribute the remaining noise at zero K⁺ flux in the standard solution (at $V_m = E_K$ in Fig. 3) to calcium and nonspecific leakage currents. Metabolism-dependent processes most likely do not contribute to the membrane noise since cooling the specimen to 4 °C did not significantly alter noise spectra.

To relate membrane noise to spontaneous ciliary reversal a low-potassium bathing solution was chosen (1 mM KCl, 1 mM CaCl₂, 1 mM Tris, pH 7.2). In this solution the noise intensity at resting membrane potential (-25 to -29 mV) was substantially increased, as has to be expected from the mentioned relationship between noise intensity and K⁺ flux, since in this case the steady K⁺ flux is larger. Spontaneous

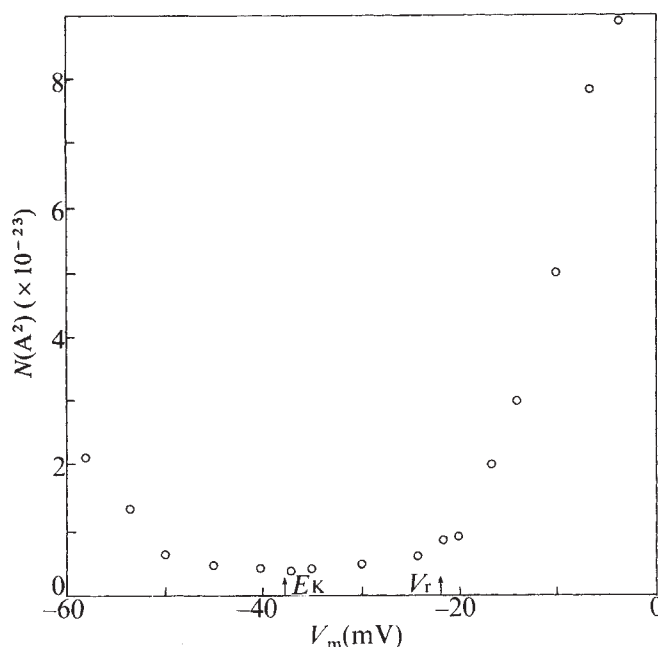


Fig. 3 Intensity N of $1/f$ noise plotted against membrane potential V_m . N was determined as the value of $S_I(f)$ at 1 Hz. Resting potential $V_r = -22$ mV, K⁺-equilibrium potential $E_K = -38$ mV in the standard solution.

transient ciliary reversals did occur, coinciding with depolarisations ranging from about 3–8 mV in amplitude and about 0.1–1 s in duration. These random depolarisations caused the noise to be non-stationary, which implies that the usual noise processing techniques do not give meaningful results. Under voltage clamp, on the other hand, spontaneous reversals were not observed. In this case noise current was stationary and exhibited a $1/f$ spectrum (intensity $N = 5 \times 10^{-23}$ A² at resting membrane potential).

We conclude therefore that membrane $1/f$ -noise voltage,

generated mainly by the steady passive K^+ flux, can activate Ca^{2+} conductance occasionally and thus underlies spontaneous reversals in *Paramecium*.

To our knowledge this is the first report which provides experimental evidence for a connection between membrane $1/f$ noise and simple behavioural effects in a living organism.

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Antagonistic effects of GABA and benzodiazepines on vestibular and cerebellar neurones

ALTHOUGH benzodiazepines are widely used psychotropic drugs their mode of action within the central nervous system (CNS) is controversial^{1–4}. Based on biochemical and pharmacological investigations of various central and peripheral neural substrates, Costa *et al.*⁵ and Haefely *et al.*⁶ suggested that they act, at least in part, through a facilitation of γ -aminobutyric acid (GABA)-ergic transmission. We have tested this hypothesis electrophysiologically at the single unit level in areas of the CNS in which GABA has been unequivocally shown to be an inhibitory transmitter. Single unit recordings were made in neurones of the lateral vestibular nucleus (Deiters) and in Purkinje cells of the cerebellum.

Deiters neurones receive a monosynaptic GABA-ergic inhibitory input through Purkinje cell axons originating in the cerebellar cortex. There is conclusive evidence for the synaptic release of GABA at the inhibitory afferences of Deiters neurones^{7–9}. Furthermore, GABA is released at the level of the inhibitory synapses between the so-called basket cells and the Purkinje cells¹⁰. Purkinje cells were identified on the basis of the spontaneous ‘inactivation response’¹¹ recorded at the appropriate levels of electrode penetration.

Experiments with Deiters neurones involved three cats anaesthetised with N_2O and artificially ventilated. Deiters neurones were activated by antidromic stimulation of the vestibulo-spinal tract (Fig. 1a). The antidromic response was inhibited by the preceding stimulation of Purkinje cell axons (Fig. 1b). Intravenous diazepam had no effect on the antidromically evoked action potential (Fig. 1c), but readily and reproducibly suppressed monosynaptic inhibition of Deiters neurones evoked by stimulation of the cerebellum (Fig. 1b). The inhibitory effect of the stimulation of Purkinje fibres (Fig. 1b) could be duplicated by the iontophoretic application of the inhibitory neurotransmitter GABA on to Deiters neurones; antidromically evoked action potentials were reduced by GABA in a

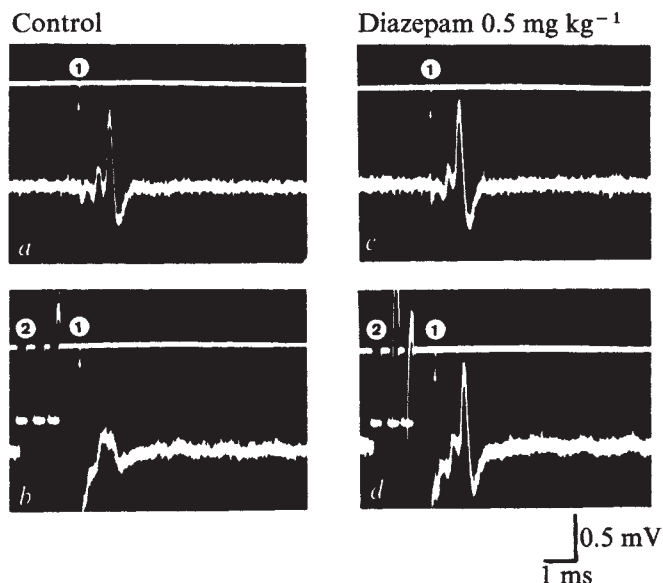
dose-dependent manner. Likewise, the intravenous injection of diazepam suppressed this inhibition of the antidromically evoked potential by GABA.

The inhibitory effect of Purkinje cell fibres on Deiters neurones, which was abolished by diazepam (Fig. 1d) could be restored either by increasing the intensity of stimulation of Purkinje fibres, or by increasing the amount of microiontophoretically delivered GABA. The effect of increased cerebellar stimulation was in turn antagonised by locally applied bicuculline (compare ref. 12).

The experiments on Purkinje cells were carried out on five Füllinsdorf albino rats (200–300 g body weights) anaesthetised with urethane (1.2 g kg^{-1} , intraperitoneally). In all cases, the spontaneous firing of Purkinje cells could be inhibited completely and instantaneously by small amounts of iontophoretically applied GABA (of the order of 2–5 nA). This inhibition was strikingly affected by a preceding injection of the benzodiazepine derivative bromazepam: a large amount of GABA (10 nA) now required several seconds to suppress spontaneous activity (Fig. 2a). The same amount of locally applied GABA could be made even less effective in suppressing neuronal activity by increasing the dose of injected bromazepam (Fig. 2b). This indicated again a dose-dependent relationship between the effects of GABA and the benzodiazepine derivative.

These electrophysiological findings on vestibular and cerebellar neurones demonstrate for the first time a functional antagonism between benzodiazepine derivatives and the inhibitory neurotransmitter GABA. In both systems, inhibitory GABA influences are suppressed in a dose-dependent manner by these psychotropic drugs, but their effect can be overcome by increasing local GABA concentrations, either through stimulation of the appropriate axons or by microiontophoretic delivery of GABA. By contrast, the effect of the specific GABA antagonist, bicuculline, does not seem to be impaired by benzodiazepine. To ascertain that the benzodiazepine derivative diazepam

Fig. 1 Modification of antidromic (1) potentials recorded from a single neurone in the Deiters nucleus of the cat. *a*, Potential obtained of the antidromic stimulation of the vestibulo-spinal tract with single pulses of 0.5 mA, 0.05 ms duration (five responses superimposed). *b*, Abolition of the antidromic potential by preceding orthodromic stimulation (2) (train of three pulses, 0.8 mA, 0.1 ms, 250 per s) of Purkinje fibres. *c*, Antidromic response obtained 6 min after intravenous injection of diazepam (0.5 mg kg^{-1}) is unchanged. *d*, Suppression of the inhibitory effect of the orthodromic stimulation (compare *b*) after injection of diazepam (5 min).



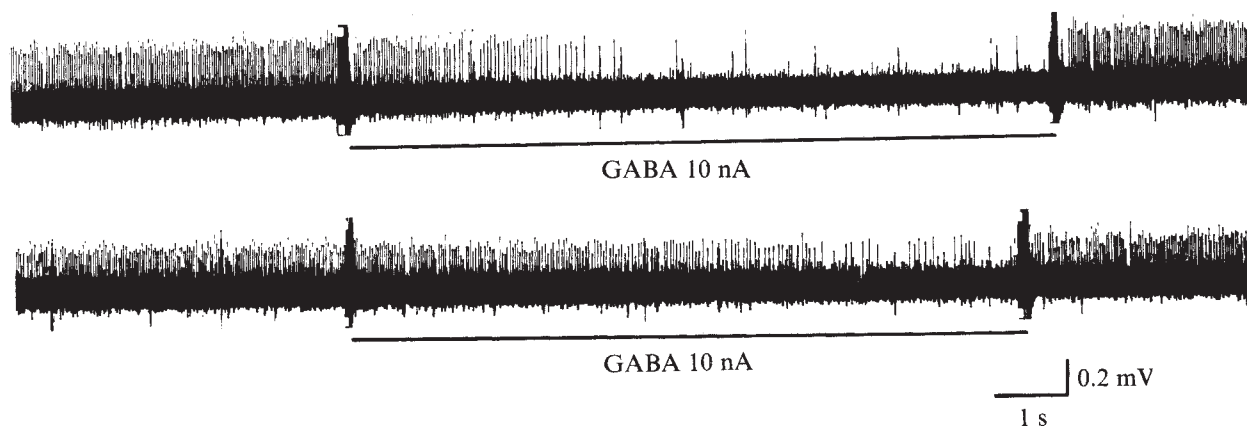


Fig. 2 Single unit activity of a rat Purkinje cell recorded through the central barrel of a five-barrelled micropipette¹³; GABA was delivered from a lateral barrel as a 0.5–1 M solution at pH 3.0–3.5. *a*, Delayed inhibitory effect of GABA (10 nA) 47 min after intravenous injection of bromazepam (2 mg kg⁻¹). Action potentials persisting during the second two-thirds of GABA iontophoresis represent the non-suppressible climbing fibre responses¹⁴. *b*, Further attenuation of the GABA effect (10 nA) 2 min after a second intravenous injection (4 mg kg⁻¹).

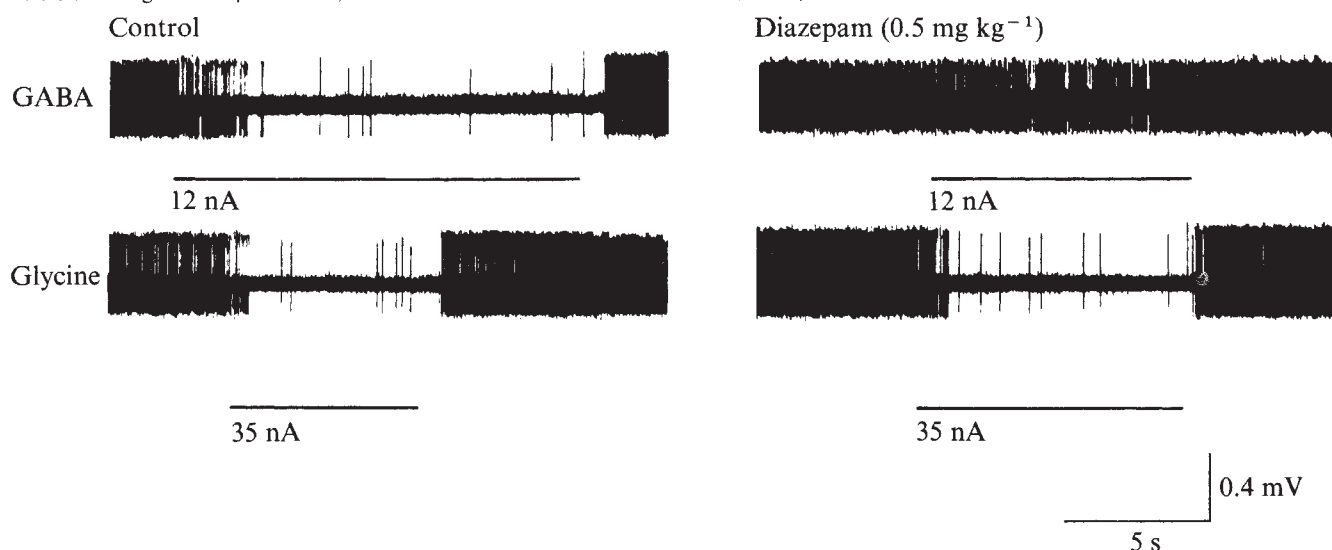


Fig. 3 Single unit activity of a cat Purkinje cell. GABA and glycine were delivered alternately by iontophoresis (12 nA and 35 nA respectively, indicated by horizontal bars) at pH 3.0 from 0.5 M solutions. After intravenous injection of diazepam (0.5 mg kg⁻¹) the GABA depression of cell activity (control) was nearly abolished. In contrast, the glycine-induced depression was not reduced after injection of drug.

specifically counteracts GABA-ergic transmission its effect on glycine-induced inhibition³ was tested in cat Purkinje cells (Fig. 3). These neurones were less sensitive to glycine and intravenously administered diazepam had no antagonistic influence in the inhibitory effect seen after the iontophoretic delivery of glycine. Thus, this study on Purkinje cells suggests differential effects of benzodiazepines on the two inhibitory amino acids, GABA and glycine. In further experiments, the soluble benzodiazepine derivatives, chlordiazepoxide HCl, was applied iontophoretically to Purkinje cells in rats and cats (unpublished results). Again, the GABA-mediated inhibition of Purkinje cells by basket cells, as well as the direct inhibitory effects of GABA were attenuated or abolished.

In summary, these results fail to support the idea that the action of benzodiazepine could be explained by a facilitation of the GABA-ergic transmission^{5,6}. On the contrary, the GABA-mediated inhibition on Deiters neurones and Purkinje cells was clearly antagonised by these drugs.

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Differentiation of L-aspartate and L-glutamate high-affinity binding sites in a protein fraction isolated from rat cerebral cortex

THE possibility that L-glutamate and L-aspartate are excitatory transmitters in the CNS is supported by neurochemical and neurophysiological evidence¹. The problems of identifying particular synapses that are excited by one or the other of these dicarboxylic amino acids, and distinguishing

Table 1 Inhibition of the binding of L-¹⁴C-glutamate and L-¹⁴C-aspartate to the hydrophobic protein from rat cerebral cortex by L-glutamate inhibitors

	Bound L- ¹⁴ C-glutamate (nmol per mg protein)	Bound L- ¹⁴ C-aspartate (nmol per mg protein)
Control	0.56 ± 0.03	1.98 ± 0.28
DL-α-methyl glutamate	0.29 ± 0.04	1.54 ± 0.19
Diethylester of L-glutamate	0.19 ± 0.06	1.46 ± 0.10
L-Nuciferine	0.26 ± 0.09	1.62 ± 0.07

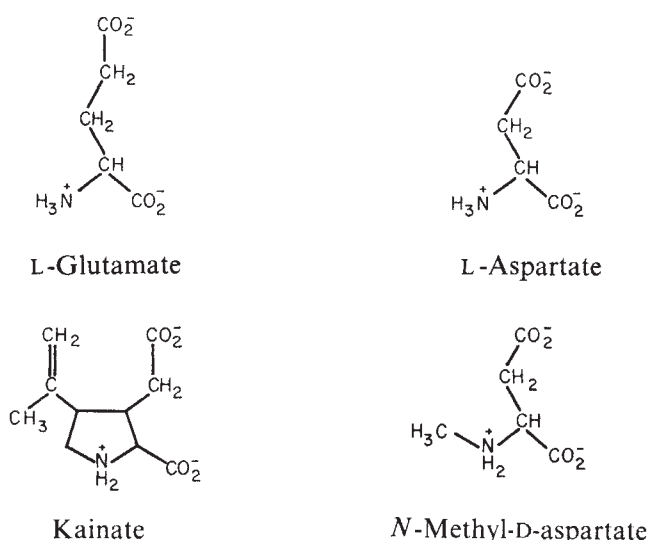
The binding was carried out in aliquots of 100 µg of protein from the first fraction separated by Sephadex LH20 chromatography of the rat cerebral cortex. In the control experiments the binding was carried out with 2×10^{-7} M L-¹⁴C-glutamate or 4×10^{-7} M L-¹⁴C-aspartate. After incubation for 15 min the samples were rechromatographed on small Sephadex LH20 columns (15 × 0.8 cm) and the bound radioactivity and protein, eluted in chloroform, were measured. For the competition experiments the fractions were preincubated for 30 min with 4×10^{-5} M of the various drugs. The results are the mean of three experiments ± s.d. L-(U)-¹⁴C-aspartate (231 mCi nmol⁻¹) and L-(U)-¹⁴C-glutamate (270 mCi nmol⁻¹) were from Amersham.

between the two receptors are, however, more difficult. At the recent International Meeting of Neurochemistry (September 1975) Graham summarised some of the evidence favouring distinct functions for the two amino acids, concentrating on the possible transmitter role of L-aspartate. For example, a higher content of this amino acid is found in the ventral than in the dorsal grey matter of the spinal cord², aortic occlusion leads to loss of aspartate content and degeneration of small neurones³, reduction in aspartate content is seen in the spinal cord after section of dorsal roots⁴ and in the olfactory cortex after olfactory bulbectomy⁵, which also decreases glutamate. Renshaw cells are slightly more sensitive to the iontophoretic administration of L-aspartate while the reverse is true of interneurons of the dorsal spinal horn⁶. It is possible that L-glutamate and L-aspartate, being flexible molecules, fold or unfold and could interact with either receptor and McCulloch *et al.*⁷ accordingly used kainate, a conformational restricted analogue of glutamic acid, which would be unlikely to fold to occupy the aspartate receptor, and N-methyl-D-aspartate, which would be too small to interact with the glutamate receptor (Fig. 1). The difference in sensitivity between the two groups of neurones to these agonists was much greater: the Renshaw cells were much more sensitive to N-methyl-D-aspartate and the dorsal interneurons to kainate⁷.

The L-glutamate binding proteins of insect⁸ and crustacean muscle^{9,10} are hydrophobic proteins (that is, proteolipids) extractable with organic solvents. Such proteins did not bind L-aspartate and, in the case of the crustacean muscle, can be separated from another protein binding γ-aminobutyric acid (GABA)^{9,11}. Recently we have separated a hydrophobic protein fraction from synaptic membranes of the rat cerebral cortex that binds, with high affinity, L-glutamate and GABA¹². The same protein fraction also binds L-aspartate and this poses the problem of differentiating between the various binding sites. We shall present here some evidence that this fraction contains a protein binding L-aspartate and which may be its receptor.

Amounts of 200–300 mg of lyophilised cerebral cortex of adult rats were extracted with chloroform-methanol

(2:1)¹³ and the extract, containing 3–4 mg of hydrophobic protein (that is, proteolipid) per g fresh tissue, was chromatographed on a Sephadex LH20 column equilibrated with chloroform. Elution was carried out with chloroform and a series of mixtures of chloroform-methanol of increasing polarity¹⁴. The first protein fraction, eluted in chloroform in the void volume of the column, is the one that binds L-¹⁴C-aspartate and L-¹⁴C-glutamate. The binding sites were differentiated by the following experiments. (1) Aliquots of the first protein fraction, containing 100 µg of protein, were preincubated with 4×10^{-5} M of L-glutamate or with 2×10^{-5} M L-aspartate; then they were submitted to binding with 4×10^{-7} M L-¹⁴C-aspartate or 2×10^{-7} M L-¹⁴C-glutamate following an experimental procedure similar to that indicated in Table 1. It was found that L-aspartate did not inhibit the binding of L-¹⁴C-glutamate (0.41 nmol per mg protein in the control and 0.43 ± 0.02 in the experimental) and, vice versa, L-glutamate only slightly inhibited the binding of L-¹⁴C-aspartate (3.07 nmol per mg protein in the control and 2.62 ± 0.07 in the experimental).

**Fig. 1** Structural formulae of L-glutamate and L-aspartate and their respective analogues: kainate and N-methyl-D-aspartate.

(2) Experiments were carried out with the physiological competitors of L-glutamate. DL-α-methyl glutamate¹⁵, the diethylester of L-glutamate¹⁵ and L-nuciferine¹⁶ were much more potent in blocking the binding of L-¹⁴C-glutamate than in blocking that of L-¹⁴C-aspartate (Table 1). The three drugs inhibited, respectively, by 65, 48 and 54% the binding of L-¹⁴C-glutamate and only by 26, 22 and 18% that of L-¹⁴C-aspartate.

(3) The most striking results were, however, those obtained with the two agonists kainate and N-methyl-D-aspartate, which according to McCulloch *et al.*⁷ bind preferentially glutamate and aspartate receptors respectively (Fig. 1). Kainate inhibits the binding of L-¹⁴C-glutamate by 54% but it does not interfere with the binding L-¹⁴C-aspartate (Table 2). On the other hand, N-methyl-D-aspartate does not change the binding of L-¹⁴C-glutamate but inhibits that of L-aspartate by 45%. These results are in

Table 2 Inhibition of the binding L-¹⁴C-glutamate and L-¹⁴C-aspartate to the hydrophobic protein from rat cerebral cortex by their analogues kainate and N-methyl-D-aspartate

Labelled amino acid	Control		Kainate (2.5×10^{-5} M)		N-Methyl-D-aspartate (4×10^{-5} M)	
	(nmol per mg protein)	(nmol per mg protein)	(nmol per mg protein)	Inhibition %	(nmol per mg protein)	Inhibition %
L- ¹⁴ C-Glutamate (2×10^{-7} M)	0.56 ± 0.03	0.26 ± 0.02	0.26 ± 0.02	54	0.57 ± 0.11	0
L- ¹⁴ C-Aspartate (4×10^{-7} M)	1.98 ± 0.28	1.99 ± 0.19	1.99 ± 0.19	0	1.09 ± 0.07	45

The binding was carried out as described in Table 1. The results are the mean of three experiments ± s.d. Kainate and N-methyl-D-aspartate were generous gifts from Professor D. R. Curtis.

agreement with the physiological observations of McCulloch *et al.*⁷, and support their interpretation¹⁷.

Our findings suggest that the hydrophobic protein fraction isolated from the cerebral cortex, in the void volume of the Sephadex LH20 column, contains different binding sites for L-aspartate and L-glutamate. We have previously shown that it also contains different binding sites for GABA¹². It remains to be seen whether these binding sites reside in a single or, more probably, in different proteins and whether they represent the true receptors or belong to the active transport system for these amino acids^{18,19}. In favour of their possible receptor function is the fact that, in our experiments, the binding is carried out in the absence of Na ions, which are essential for the transport mechanism to operate. Furthermore, several of the drugs used, such as N-methyl-D-aspartate, kainate and diethyl ester of L-glutamic acid are not substrates for the transport system¹⁹.

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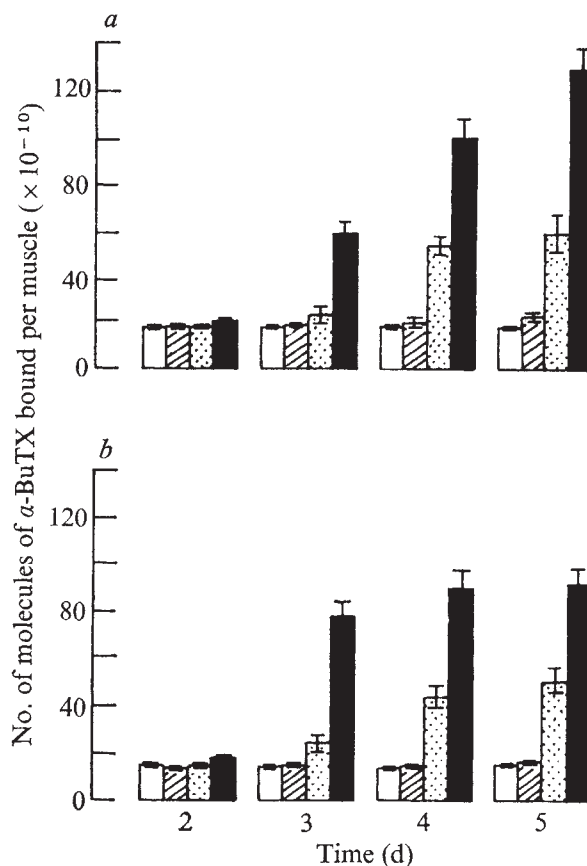
Comparison of α -bungarotoxin binding to skeletal muscles after inactivity or denervation

DENERVATION of skeletal muscles causes a spread of acetylcholine (ACh) sensitivity along muscle fibres, but it is not yet clear whether this results from muscle inactivity or from a loss of trophic influence of the nerve on the muscle¹. It has been suggested that inactivity is responsible for the phenomenon because the application of a cuff containing a local anaesthetic to a nerve blocked the conduction of the nerve impulses and caused the development of widespread sensitivity to ACh (ref. 2, but see ref. 3). The local anaesthetics used in that study, however, have since been shown to block axoplasmic transport *in vitro*^{4,5} and *in vivo*⁶, as well as to induce neuronal degeneration⁷. Axoplasmic transport seems to be involved in the trophic influence of the nerve on muscle because blocking this transport induces spreading of ACh sensitivity in muscle fibres^{8,9}. We have used tetrodotoxin (TTX), incorporated into silicone cuffs placed around motor nerves, to test whether the resultant inactivity induced a spread of ACh sensitivity in skeletal muscle, as inferred from the binding of α -bungarotoxin (α -BuTX). TTX was chosen because it does not block axoplasmic transport *in vitro*⁹.

Female Sprague-Dawley rats (225–250 g) were anaesthetised with ether, the left sciatic nerve was exposed, and a hollow cylindrical cuff made from Silastic 382 (Dow Corning) was placed around the nerve. The control cuffs contained 20% NaCl (w/w) mixed in the Silastic, and the TTX cuffs contained in addition 0.02% TTX (Sigma); each TTX cuff contained approximately 5 μ g of TTX. Paralysis was evaluated three times a day by inspecting the toe-spreading reflex when the animals were lifted by the tail⁷; paralysis was present when the animals that received a TTX cuff recovered from anaesthesia and lasted 3–5 d, but the animals that received a control cuff showed no paralysis. The muscles of the contralateral side served as unoperated controls in the animals that received a control cuff, while those contralateral to a TTX cuff were denervated by removing a 5-mm segment of the sciatic nerve in the mid-thigh region.

At various times thereafter, the extensor digitorum longus (EDL) and soleus (SL) muscles from both sides were removed under ether anaesthesia and exposed to ¹²⁵I- α -BuTX¹⁰. The muscles were incubated for 1 h (22 °C) in a Krebs solution containing labelled α -BuTX (1 μ g ml⁻¹), and then washed for 16–20 h with Krebs solution (4 °C). The total radioactivity retained by the muscles was measured (Nuclear Chicago gamma spectrometer), and the change in α -BuTX binding induced by control cuffs, TTX cuffs and surgical denervation was compared (Fig. 1). The muscle inactivity produced by the TTX cuffs resulted in only a slight increase in α -BuTX binding after 3 d, but α -BuTX binding increased fivefold in the SL and threefold in the EDL muscles denervated for the same time; the difference of α -BuTX binding to the TTX-inactivated

Fig. 1 Effect of TTX cuffs, control cuffs, and surgical denervation on the binding of α -BuTX to the EDL (a) or SL (b) muscle of the rat. Each column represents the mean $\pm$ s.e. of 7–11 muscles. Binding is expressed as molecules of α -BuTX bound, which was calculated from the specific activity of the ¹²⁵I- α -BuTX used. Columns: open, unoperated; hatched, control cuff; spotted, TTX cuff; black, denervated.



muscles and the denervated muscles was significant ($P < 0.001$). But muscles that had been paralysed for 4 and 5 d by TTX cuffs showed a clear increase in α -BuTX-binding ($P < 0.001$), although the magnitude of this increase was again less ($P < 0.001$) than that produced by denervation. Some of the animals that had been exposed to TTX cuffs for 4 and 5 d showed some recovery of function and it is possible that this short (< 12 h) recovery reversed some of the induced increase of ACh receptors; however, its effect was probably small because α -BuTX binding was still increased ($240 \pm 30\%$ of control) in the muscles of animals 3-4 d after recovery from 4-5 d of TTX-induced inactivity.

The increased α -BuTX binding observed with the TTX cuffs was not due to nerve degeneration. Rats that had received cuffs 3 d earlier were anaesthetised with pentobarbital (intraperitoneal, 50 mg kg^{-1}), the sciatic nerve was exposed and stimulated electrically (supramaximal voltage, 0.3 ms, 0.4 Hz), and the isometric contractions of the EDL muscle were recorded; the muscle was also stimulated directly. In nine animals in which impulse conduction was completely blocked by a TTX cuff, nerve stimulation distal to the cuff induced a muscle contraction ($11.4 \pm 0.7 \text{ g}$ tension per 100 mg muscle wet weight; mean $\pm$ s.e.) similar to that produced by direct muscle stimulation ($11.9 \pm 0.7 \text{ g}$): thus, TTX, unlike lidocaine⁷, does not induce nerve degeneration. In 10 animals that received a control cuff, nerve stimulation proximal or distal to the cuff, and direct muscle stimulation all induced similar muscle contractions (10.8 ± 0.6 , 11.6 ± 0.5 , $11.5 \pm 0.4 \text{ g}$ tension, respectively), showing that the cuff itself did not damage the nerve.

Other results demonstrated that the TTX cuffs did not block axoplasmic transport *in vivo*. For these experiments a ligature (Ethicon 4-0 thread) was tied around the sciatic nerve 3-4 mm distal to the cuffs and the accumulation of cholinesterase (ChE) activity at these ligatures after 20 h (ref. 11) was measured. Nerve segments (2 mm) were weighed, homogenised in ice-cold sucrose (0.32 M; 20 mg tissue per ml) and the ChE activity in $50 \mu\text{l}$ of the homogenate was assayed (22°C) by the method of Ellman *et al.*¹², using acetylthiocholine ($200 \mu\text{M}$) as the substrate. There was no significant difference ($P > 0.20$) between the accumulation of ChE activity at a ligature distal to a TTX cuff and the accumulation in ligatured control nerves ($289 \pm 22\%$ of penultimate segment; mean $\pm$ s.e.), whether the ligature was tied 1 h ($306 \pm 22\%$) or 24 h ($271 \pm 27\%$) after placing the TTX cuff.

Thus, our results show that local application of TTX to the sciatic nerve of rats will produce muscle inactivity without causing nerve degeneration or blocking axonal transport. This inactivity increases the amount of α -BuTX bound by the SL and EDL muscles, but this change seems to be less than and to have a different time course from that induced by denervation. This difference might reflect a role for factors other than activity in the control of ACh sensitivity in skeletal muscles.

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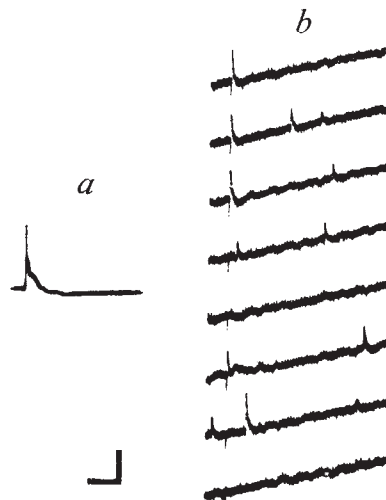
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Suppression of transmission at foreign synapses in adult newt muscle involves reduction in quantal content

IN the search for the principles underlying the orderly development of the nervous system, it is interesting to know whether vertebrate neurones or muscle fibres can distinguish synaptic contacts of the axons which normally innervate them from the input of incorrect (or foreign) nerves. This possibility has been considered by asking whether foreign synapses induced on a denervated muscle fibre are displaced or suppressed when the muscle is re-innervated by its normal nerve. Observations of oculomotor reflexes in goldfish after experimental cross innervation of extraocular muscles led to the proposal that correct re-innervation suppressed foreign synapses¹. Attempts to reproduce these results in the same system², however, led to the opposite conclusion—that foreign transmission continued unabated after reinnervation by the correct nerve. A similar persistence of foreign synaptic function was found in reinnervated perch gill muscle³, frog fast muscle⁴, mammalian skeletal muscle^{5,6} and sympathetic ganglion cells⁷. Adult salamander muscle is the one other system in which suppression of incorrect synapses has been indicated; by the criterion of muscle fibre contraction monitored during foreign innervation and correct reinnervation, Cass *et al.*⁸ concluded that return of the correct nerve caused inactivation of foreign synaptic input. The present investigation examines this phenomenon at the level of transmitter

Fig. 1 Responses from a single anconeus muscle fibre 50 d after correct nerve resection. *a*, Single stimulus to the correct nerve evoked a large endplate potential with action potential superimposed. *b*, Consecutive responses to repeated maximal stimulation of the foreign nerve at a frequency of 0.2 per s. One stimulus pulse occurs near the beginning of each sweep, as indicated by artefact. With some stimuli the foreign nerve failed to release transmitter (traces 4, 5, 7 and 8 from top). Note the spontaneous miniature endplate potentials occurring randomly in (*b*). Calcium concentration normal (1.8 mM) in both. Calibration marks 40 mV and 100 ms in (*a*), 2 mV and 400 ms in (*b*). All five fibres studied in the muscle had been reinnervated by the correct nerve and had low quantal contents for foreign transmission ($m = 0.66, 0.94, 1.06, 0.43, 1.47$).



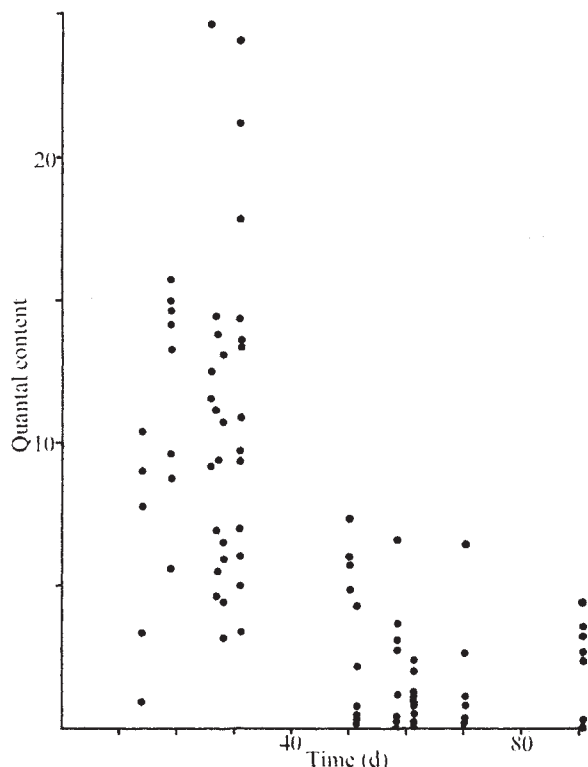


Fig. 2 Scatter diagram of mean quantal content of foreign nerve release plotted as a function of time after the correct nerve was resected. Each point represents the mean content at a single anconeus fibre, measured as indicated in the text. Correct reinnervation was first detected 26 d after resection, and was completed within 50–60 d. In some cases movement artefacts resulting from stimulation of the correct nerve prevented us from determining the absolute amplitude of the correct junctional potential. Because of variability in the rate of correct nerve regrowth, we do not know the time elapsed since correct reinnervation of any given muscle fibre. Not represented in this figure are four muscles, examined at 57, 70, 93, 135 d, in which no foreign nerve responses were found.

release from individual synapses, and provides evidence that transmission by foreign nerve terminals declines in quantal content when adult newt muscle is reinnervated by its correct nerve.

Adult newts, *Notophthalmus viridescens*, were anaesthetised with tricaine (1/1,000 in Ringer solution). The forelimb flexor (humero-antibrachialis) muscle was excised completely and its nerve was displaced laterally to the surface of the antagonistic anconeus muscle. The animals were then held in tanks of water and fed weekly on liver or brine shrimp. Two weeks after the initial operation the correct nerve to the anconeus muscle was crushed, or cut and resected. In some animals the correct nerve was crushed a second time 2 or more weeks after the initial denervation. At various times after the final denervation intracellular recordings were made from single fibres of the anconeus muscle in normal amphibian Ringer, with independent stimulation of the correct and foreign nerves.

The quantal content of the foreign transmission was determined in individual fibres from the coefficient of variation of the evoked responses⁹, obtained by stimulating 200 times at a frequency of 0.2 per s. In 25 fibres this value for quantal content was compared with that obtained by the method of failures⁹; there was no significant difference between the values determined by these two means.

Muscles examined within the first week after lesion of the correct nerve showed no functional transmission from either nerve. Between the first and second week transmission from the foreign nerve was detected in a few fibres; in these, stimulation evoked endplate potentials of low amplitude, with frequent failures. The longer the period of

correct denervation, the greater was the extent and efficacy of foreign innervation. Thus, when the correct nerve was cut rather than crushed initially, or when it was crushed a second time 10–14 d after the first denervation, a greater proportion of the fibres developed functional foreign transmission and the average amplitude of the responses evoked by foreign nerve stimulation was larger. In some fibres the amplitude of foreign response became large enough to cause an action potential and twitch, like that seen in normally innervated adult fibres^{10,11}.

Approximately 2 weeks after the correct nerve was crushed, or 3–4 weeks after it was resected, its axons began to reinnervate the muscle. At this stage individual fibres were found which received functional contact from both correct and foreign axons. The correct synapses rapidly developed to their normal state of transmission since correct input, when detected, was almost always sufficiently strong to cause normal action potentials (Fig. 1a). Within 3 months after the final denervation all fibres had received correct reinnervation.

The synaptic potentials resulting from foreign transmission were usually subthreshold in those fibres which had received correct reinnervation (Fig. 1b). The mean quantal release from the foreign nerve was measured in individual fibres at various times after correct nerve resection. The pooled results from 84 fibres of 13 muscles in which the correct nerve had been resected or crushed twice are presented in Fig. 2. These data reveal that foreign transmission declines in quantal content with time after the correct nerve returns. A similar series of experiments in which the correct nerve was crushed only once gave the same general result, although the maximum values of foreign quantal content were lower and the decline occurred more rapidly.

Although correct reinnervation resulted in a decline in the amount of transmitter released per impulse from foreign terminals, the average size of the quantal events remained unchanged; release failure was frequent yet the mean amplitude of foreign evoked quanta was normal (0.5–2 mV) and equal to that of spontaneous potentials in the same fibre, as illustrated in Fig. 3 (presumably the spontaneous events result from release by correct and foreign terminals). This indicates that the suppression of foreign inputs involves a decline only in the number of packets of transmitter released, and not in the amount of transmitter per packet nor in the sensitivity of muscle membrane to transmitter at the site of foreign nerve contact.

One potential difficulty with such physiological analysis arises from the fact that not all the fibres in the anconeus muscles can be assumed to receive innervation from foreign axons. Thus, after correct reinnervation a complete lack of response to foreign nerve might result either because a fibre had never received foreign innervation or because such innervation had been suppressed. But this does not seem to present a real problem in our experiments: in the set of animals with correct nerve resection, all muscles ($n=10$) examined in the first 2 months had some fibres with foreign transmission whereas in only half (4/8) of those studied 2–4 months after denervation could fibres with foreign transmission be found.

In six animals the correct nerve was recrushed 6 months after the first lesion; foreign transmission reappeared within as little as 2 days, and developed sufficient strength to elicit action potentials within 1 week. This rapid return of foreign transmission, as compared with the 10–14 d delay preceding the initial establishment of foreign innervation, suggests that foreign synapses, suppressed on correct reinnervation, may remain in close proximity with the muscle fibres.

Neither the morphology nor the relative position of correct and foreign synapses along individual muscle fibres is known. In normal muscles single fibres are innervated

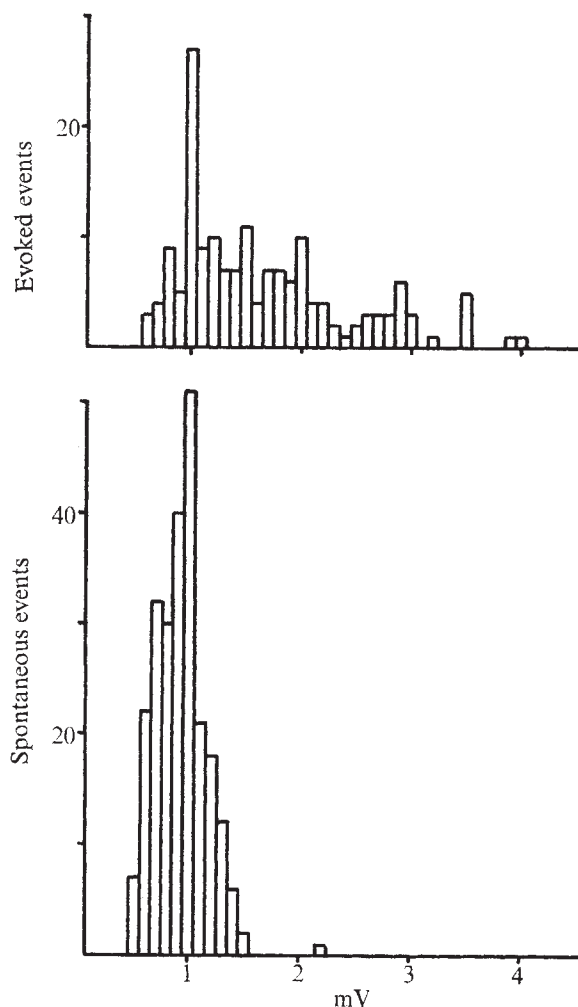


Fig. 3 Histograms comparing the amplitudes of spontaneous miniature endplate potentials (top) and of responses evoked by foreign nerve stimulation (bottom) in the muscle fibre from which examples in Fig. 1 are taken. The foreign nerve was stimulated 251 times, with 87 failures: $m = 1.1$. The calcium concentration was normal (1.8 mM). Noise level, 100 μ V.

at several sites along their length, as revealed by electrophysiology^{10,11} and by cholinesterase staining (unpublished). This suggests that here correct and foreign synapses occur in close proximity to one another.

Our results indicate that muscles of the adult newt differ from those of higher vertebrates in that they can discriminate between correct and incorrect innervation, and suppress the incorrect synaptic input. Urodele amphibia, with the ability to regenerate limbs, may retain in adulthood a capacity usually confined to embryos which permits them to correctly reconnect their peripheral innervation.

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Note added in proof: The proportion of anconeus fibres with functional foreign synapses has been determined at short and long periods after correct nerve resection. In eight muscles examined 19–47 d after resection all of 176 fibres received foreign input, whereas in nine muscles 57–135 d after resection only 37 of 151 fibres (25%) had functional foreign input.

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Effect of muscle disuse on acetylcholine receptors

MOTOR nerves have an important role in the trophic regulation of many properties of skeletal muscle, including acetylcholine receptor (AChR) distribution¹. In innervated muscle, the density of AChR at neuromuscular junctions is at least 2,500 times greater than at extrajunctional regions². When the motor nerves are sectioned, extrajunctional AChR density increases markedly^{3–5}. The trophic mechanisms by which the nerves normally prevent this denervation change are not yet well understood. It has been suggested that muscle use contributes to the control of AChR. This hypothesis has been tested in two ways. First, electrical stimulation was applied directly to denervated muscle, and the extrajunctional ACh sensitivity was determined. The results indicated that stimulation-induced use largely prevented the denervation effect^{6,7}. Second, attempts have been made to assess the effect of disuse of muscle on extrajunctional ACh sensitivity. Various methods have been used to eliminate muscle contraction but all have had shortcomings. In some cases, the 'disuse' has been incomplete^{8–10}; in others, the methods used to block nerve conduction^{7,11} have produced blockade of axonal transport^{12,13}, which may itself result in denervation effects^{14,15}. Using a new model of disuse free of these problems, we have now compared the effects of disuse and denervation with respect to extrajunctional AChR density. We report here evidence that disuse produces a significant increase in extrajunctional AChR, but does not completely reproduce the effect of surgical denervation.

In our model, complete blockade of nerve conduction, without alteration of other nerve functions, has been produced by repeated local application of tetrodotoxin (TTX) to the sciatic nerves of rats. TTX interferes with conduction of electrical impulses in excitable membranes by blocking sodium conductance channels¹⁶. Quantitative determinations of extrajunctional AChR density in disused and denervated muscles have been made by means of a technique using ¹²⁵I- α -bungarotoxin binding¹⁷.

Experimental procedures were carried out on Sprague-Dawley female rats (190–215 g) under chloral hydrate anaesthesia (400 mg kg⁻¹). The sciatic nerve was exposed by an incision in the thigh, and a subperineurial injection of TTX or Ringer solution was made by means of a glass micropipette coupled to a micrometre-driven syringe, mounted on a micromanipulator. In initial trials, we found that intraneural injection of 2 μ g of TTX in 2 μ l of Ringer solution produced complete paralysis beginning within 10 min, and lasting usually for more than 48 h. More detailed studies of the physiological effects of the TTX treatment were made in six clinically paralysed animals 48 h after intraneural injection. To determine whether the nerve block was complete, the sciatic nerve was stimulated *in vivo* proximal to the injection site, while electrical recordings were made at high gain from the surface of the soleus and extensor digitorum longus (EDL) muscles, and the exposed muscles were observed under the stereomicroscope. The nerve and muscle were then removed in continuity, and intracellular recordings were made *in vitro* (during nerve stimulation) with conventional 3 M KCl-filled micro-

electrodes. By these criteria, nerve conduction was totally blocked at the level of the TTX injections, although the distal portion of the nerve and the muscle functioned normally (see below).

In the experiments reported here we therefore adopted a uniform protocol in which the injections were repeated at 48-h intervals. Just before each repeat injection of TTX and at the termination of each experiment, blockade was tested by nerve stimulation. When blockade was incomplete the animals were discarded.

At 0, 4, and 7 d, the rats were anaesthetised and the EDL and soleus muscles from each were dissected out and prepared for measurement of extrajunctional AChR density. This measurement depends on the specific, quantitative and irreversible binding of ^{125}I - α -bungarotoxin with AChR. The number of bungarotoxin-binding sites is proportional (perhaps equal) to the number of AChR sites¹⁸.

The results (Table 1) show that disuse produced a significant increase in extrajunctional AChR in all groups. Denervation always produced a significantly greater increase in AChR than did disuse. The disused soleus showed significantly greater increase in extrajunctional AChR than did the comparable EDL at both 4 d ($P < 0.01$) and 7 d ($P < 0.05$).

The injection procedure itself could not account for these effects since subperineurial injections of Ringer solution did not produce an increase in AChR density above control levels. TTX did not exert an inhibitory influence on extrajunctional receptors, since treatment with denervation plus TTX resulted in levels of AChR similar to those of denervation alone.

The effects of the experimental treatments on the morphology and function of the sciatic nerve were evaluated. Nerves of all the 7-d TTX and Ringer-injected animals were embedded in Epon, and 1- μm thick sections, stained with toluidine blue, were coded and examined microscopically. No morphological differences between TTX and

Ringer-injected nerves were found; in both only occasional degenerated axons were identified. The distal segment of the blocked nerves and the muscle fibres could function normally, since stimulation distal to the TTX block resulted in brisk muscle contraction. Intracellular recordings of miniature endplate potentials showed normal frequency (soleus = $2.0 \pm 0.18 \text{ s}^{-1}$; EDL = $2.4 \pm 0.27 \text{ mV}$) and amplitude (soleus = $0.37 \pm 0.03 \text{ s}^{-1}$; EDL = $0.46 \pm 0.05 \text{ mV}$, corrected¹⁹ to 75 mV RMP), indicating that the motor nerve terminals remained intact. Fast axonal transport of protein and glycoprotein was measured after injection of ^3H -leucine or ^3H -fucose into the lumbar ventral horns. In nerves treated with TTX for 7 d the rate through the level of injection was normal ($385 \pm 34 \text{ mm d}^{-1}$)²⁰ and equal to that of the control side. Delivery of ^3H -fucose-labelled glycoproteins to the motor nerve endings was also similar in control and TTX-treated nerves, as shown by autoradiography of hindfoot lumbrical muscles 24 h after labelling of the ventral horns²¹.

Based on these observations, the TTX-blocked preparation seems to fulfil the requirements of a pure disuse model. Nerve impulses were effectively blocked throughout the experimental period, thereby eliminating all nerve-mediated muscle usage. The experimental procedure did not damage the nerves structurally, interfere with spontaneous transmitter release, or block axonal transport and delivery.

The observation of a denervation-like increase of extrajunctional AChR in disused muscles is generally consistent with previous reports⁸⁻¹⁰. Our quantitative studies demonstrate that the effect of disuse is large, but is not as great as that of denervation. This implies that some additional neurotrophic influence also contributes to the regulation of AChR. Many possible factors have been postulated, including substances carried by axonal transport^{14,15}, spontaneously released ACh²², and nerve-muscle membrane interaction^{23,24} but further studies are needed to clarify the role of each. We conclude that use has a prominent but not exclusive role in the neurotrophic regulation of extrajunctional AChR.

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Table 1 Effects of treatment on extrajunctional AChR density

Treatment	Soleus muscle		Time
	4 d	7 d	
Untreated	22 $\pm$ 2 (30) [†]		
Denervation	195 $\pm$ 16 (11)*	381 $\pm$ 39 (14)*	
TTX	96 $\pm$ 23 (5)* [†]	243 $\pm$ 46 (7)* [§]	
Ringer	22 $\pm$ 1 (3) [†]	12 $\pm$ 3 (6) [†]	
TTX + denervation	200 $\pm$ 23 (5)*		
Treatment	EDL muscle		Time
	4 d	7 d	
Untreated	10 $\pm$ 1 (36) [†]		
Denervation	168 $\pm$ 26 (11)*	356 $\pm$ 35 (13)*	
TTX	15 $\pm$ 4 (5) [†]	130 $\pm$ 33 (7)* [†]	
Ringer	6 $\pm$ 1 (3) [†]	7 $\pm$ 2 (6) [†]	
TTX + denervation	141 $\pm$ 29 (5)*		

*Greater than normal $P < 0.001$.

[†]Less than denervation $P < 0.005$.

[‡]Greater than normal $P < 0.05$.

[§]Less than denervation $P < 0.02$.

Muscles were teased longitudinally into three or four pieces, pinned at resting length and incubated for 3–4 h in modified Ham's F-12 culture medium containing 0.2–0.3 μg ^{125}I - α -bungarotoxin per ml (specific activity $2\text{--}5 \times 10^4 \text{ Ci mol}^{-1}$). The muscles were then washed thoroughly, fixed in 4% glutaraldehyde, and neuromuscular junctions were stained for acetylcholinesterase²⁵. Junction-free muscle strips were further dissected under the stereomicroscope into small bundles of 10–100 fibres. The number and length of fibres in each bundle were determined and the radioactivity was measured in a gamma counter. Frozen muscle sections stained with haematoxylin and eosin were used to compute average fibre diameters. Results are expressed as AChR per μm^2 (mean $\pm$ s.e.m.). Numbers in parentheses indicate number of muscles.

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Phospholipid exchange proteins in synaptosome and myelin fraction from rat brain

ACETYLCHOLINE stimulates the turnover of phosphatidyl-inositol (PI) and phosphatidic acid (PA) in brain *in vitro* and *in vivo*^{2,3}. It has been suggested that this effect on phospholipid metabolism is related to synaptic nerve pulse conductance^{4,5}, and that phospholipid exchange proteins transfer PI from the site of synthesis—the endoplasmic reticulum—to the synaptosomal plasma membrane⁵. Exchange proteins have been isolated from bovine brain that transfer PI to membranes deficient in this phospholipid *in vitro*⁶⁻⁸. These proteins also catalyse the transfer of phosphatidylcholine (PC) and PA, although to a lesser extent⁷. We report here the presence of phospholipid exchange proteins in the soluble protein fractions derived from a synaptosomal membrane and myelin fraction.

Myelin and synaptosomes were prepared from rat brain cerebral hemispheres according to a slightly modified centrifugation scheme used by Morgan *et al.*⁹ (legend to Fig. 1). The myelin fraction consisted mainly of irregularly formed multilamellar structures of vesicular appearance, often enclosing remnants of axonal contents (Fig. 1a). Electron micrographs of the synaptosomal fraction showed a rather homogeneous preparation of nerve-ending particles containing the characteristic synaptic vesicles and mitochondria; myelin fragments were absent (Fig. 1b).

The identity of the membrane fractions was further established by analysis of free cholesterol and total lipid phosphorus in the lipid extracts (Table 1). The values expressed in mg per mg protein compare favourably with those reported by Cuzner *et al.*¹⁵ for myelin (0.460 mg of cholesterol and 0.868 mg of phospholipid) and Lapetina *et al.*¹⁶ for synaptosomes (0.172 mg of cholesterol and 0.450 mg of phospholipid). The phospholipid-cholesterol molar ratio of 1.13 for myelin and 0.55 for synaptosomes agree with reported values¹⁷. Lipids from myelin and synaptosomes were further identified by two-dimensional, thin-layer chromatography¹⁸. Only the lipid extract from myelin contained cerebroside and sulphatides. This confirms that the synaptosome fraction is devoid of myelin, as the electron micrographs indicated.

Soluble proteins were released from the synaptosome and myelin fractions by resuspending the fractions in 1 mM phosphate buffer, pH 7.1. After centrifugation at 105,000g for 2 h, approximately 15% of synaptosomal protein and 6% of myelin protein were recovered from the membrane-free supernatants. These soluble protein fractions were assayed for phospholipid transfer activity (Table 2). Compared with the crude 105,000g supernatant from total brain, the PI and PC transfer activity per mg of soluble protein

was enriched eight- and fourfold, respectively, in the synaptosomes, and seven- and twofold in the myelin.

Because the transfer activities in the synaptosomal supernatant may not be representative of most activities found in the 105,000g supernatant, activities were compared by isoelectric focusing. The synaptosomal soluble protein fraction contains two major activity peaks at pH 5.02 and 5.34 (Fig. 2). Two major activity peaks were also observed with the 105,000g supernatant at pH 5.05 and 5.30. Each of the four peaks contained PI and PC transfer activity in a PI/PC activity ratio of approximately 2. Since the relative proportion which gave the two activity peaks was similar in both cases, the major phospholipid exchange proteins in the synaptosomes seem to be identical to those in the total brain cytosol. The relatively high transfer activities in the

Fig. 1 Electron micrographs of the myelin (a) and synaptosome (b) fraction. Myelin sedimented at 1,000g for 15 min was resuspended in sucrose-EDTA-phosphate buffer; the suspension was centrifuged at 1,000g for 5 min followed by centrifugation at 1,000g for 15 min. The latter pellet was resuspended, layered on 8.5% Ficoll-0.32 M sucrose, and centrifuged at 95,000g for 60 min in the Beckmann SW-27 rotor. The myelin fraction was collected at the 8.5% Ficoll interphase. The crude mitochondrial fraction containing the synaptosomes was sedimented and washed three times at 7,500g for 25 min (ref. 10). The synaptosomes that collected at the 9–12% and 12–16% interphases of the Ficoll gradient, were pooled. The membrane fractions were prepared for electron microscopy as described before¹¹.

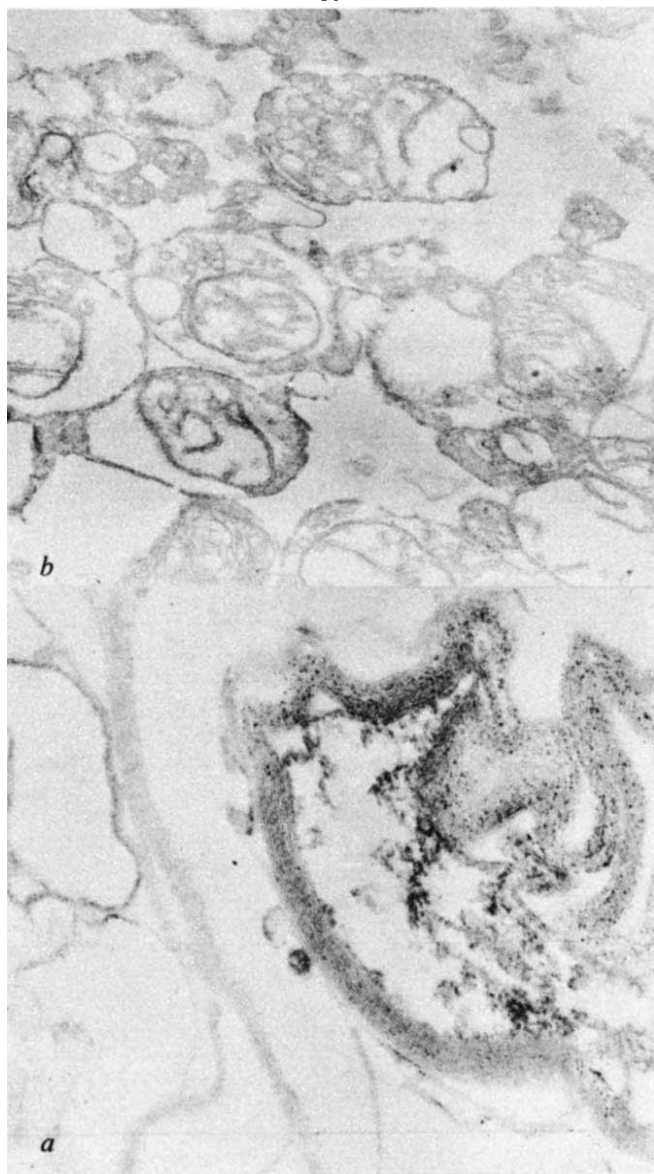


Table 1 Phospholipid and cholesterol content of membrane fractions

Lipid class	Myelin	Synaptosome
Phospholipid	0.835 ± 0.048 (4)	0.495 ± 0.026 (3)
Cholesterol	0.427 ± 0.069 (4)	0.136 ± 0.008 (3)
Cholesterol* Phospholipid	1.13 ± 0.12	0.55 ± 0.01

Lipid extracts were prepared according to a modified Folch procedure¹². Lipid phosphorus and membrane protein concentration were measured as before¹³. Cholesterol was determined by the method of Zak *et al.*¹⁴. Values are expressed in mg per mg protein. Quantities of phospholipid were obtained by multiplying the value of lipid phosphorus by 25. Numbers are given ± s.d. Numbers of experiments are given in parentheses.

*Molar ratio.

Table 2 Phosphatidylinositol and phosphatidylcholine transfer activities in membrane fractions and cytosol from rat brain

Protein fraction	PI transfer (%)*	PC transfer (%)*
Synaptosomal	28.2±4.2	7.6±1.5
Myelin	25.5±6.5	4.4±0.1
105,000g supernatant	3.7±0.4	2.0±0.1

PI and PC transfer activities were determined as before^{6,13}. The assay systems consisted of rat liver microsomal donor membranes (1.25 mg of protein) and liposomal acceptor membranes (1 µmol of egg PC, 0.02 µmol of egg PA, 0.01% (by weight) of ³H- or ¹⁴C-cholesteryl oleate). The microsomal membranes were labelled specifically with methyl-¹⁴C-PC or inositol-³H-PI. Activity is expressed as percentage of the microsomal radiolabelled phospholipid pool transferred to the liposomes, per 30 min per mg of protein at 37 °C.

*Numbers ± s.d. are based on three experiments.

synaptosomes indicate that phospholipid exchange proteins are probably intrinsic to these membrane structures.

Some nerve impulses are propagated through the synaptosomal plasma membranes by triggering the release of acetylcholine at the presynaptic site²⁰. *In vitro*, synaptosomes were found to respond to the addition of acetylcholine by an increased synthesis of PA and PI^{21,22}. *In situ*, however, the effect on PI was observed throughout the neuronal cell^{23,24}. This may be due to phospholipid transfer activities, present in the neurone, which would mask any localised effect by continuously distributing PI for example between the subcellular membrane structures (see also ref. 5).

Synaptosomal plasma membranes cannot synthesise their constituent phospholipids²⁵. It is not known to what extent the turnover of PI in this membrane is associated with the transport of this phospholipid from its site of synthesis—the intraterminal smooth endoplasmic reticulum and/or the endoplasmic reticulum in the nerve cell body down the axon. It is assumed that phospholipid exchange proteins are involved and have a role in maintaining the phospholipid composition of a membrane⁸.

The transfer activity in the soluble fraction from myelin suggests that exchange proteins are present in the axoplasm, part of which is preserved during the isolation of the myelin fraction. These proteins could be involved in the transport of phospholipids from the neuronal cell body to the nerve endings. Axonal transport of phospholipids has been demonstrated in rabbit nerves²⁶. Studies with the dorsal root ganglion of the frog have provided evidence for simul-

taneous axonal movement of PC and protein²⁷. On the other hand, part of the transfer activity may have originated from glial cytoplasm occluded in the myelin fraction.

The occurrence of identical PI transfer proteins in bovine brain, heart and liver suggests that their action is not uniquely tied to processes occurring in the neuronal cell⁷.

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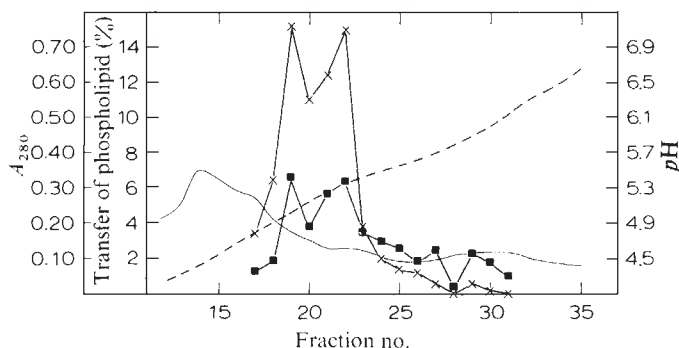
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Fig. 2 Separation of the phospholipid transfer activities present in synaptosomes, by isoelectric focusing. An LKB 8101 electrofocusing column and LKB ampholine solutions (LKB Productor AB, Stockholm) were used according to Vesterberg and Svensson¹⁹. Electrofocusing of soluble synaptosomal protein (3.7 mg) was carried out at 5 °C with a pH gradient from 4 to 8 during 5 h at 200 V followed by 64 h at 500 V; 2-ml fractions were collected. PI (×) and PC (■) transfer activity are expressed as the percentage of the microsomal radiolabelled phospholipid pool transferred per 60 min per 0.2 ml at 37 °C.



Independence of Moloney virus-induced cell-surface antigen and membrane-associated virion antigens in immunoselected lymphoma sublines

THE nature of virus-determined cell-surface antigens (CSAs), recognised by the syngeneic host on neoplastic cells induced by RNA viruses has not been properly defined in spite of much recent speculation about the possible role of cell membrane associated virion proteins¹. Lymphoma cells induced by the Moloney leukaemia virus (MLV) are said to carry MCSAs. It is a matter of some debate whether MCSA is identical with the tumour-associated transplantation antigen (TATA) detected *in vivo* by the rejection of small tumour grafts in syngeneic preimmunised animals. Views are divided on the origin and control of CSA and/or TATA: some authors regard them as virally induced, but distinct from virion antigens (see for example refs 2–5) whereas others believe that they are virion components, inserted into the cell membrane^{6,7}. Antisera against purified virion components (designated according to the recently adopted nomenclature⁸) have been used to demonstrate that p30 and gp69/71 are present on the surface of mammalian type C RNA virus-infected cells^{9–11}. We have confirmed this in the present paper using an MLV-

Table 1 Selection history of the YAC462-IR line

Passage no.	Selection	Inoculum: no. of cells	Cytotoxic sensitivity
1-3	<i>In vitro</i> * Exposure to mouse syngeneic/anti-	10^6	0.74-0.83†
4-6	MCSA serum and	10^5	0.37-0.64
7-8	rabbit complement	10^4	0.24-0.34
9-11	before each passage	10^3	0.13-0.27
12-13		10^3	0-0.10

The starting material was the YAC ascites line, 462nd serial passage.

*Mouse anti-MCSA serum was produced by the inoculation of 5×10^6 YAC cells irradiated with 6,000 r. to (A $\times$ C57 leaden) F₁ mice; 4-6 inoculations were given and serum was collected 1 week after the last inoculation. 5×10^6 cells were incubated with 0.1 ml of a 1:5 dilution of antiserum for 30 min at 37 °C. Antiserum was removed by centrifugation and 0.1 ml of a 1:5 dilution of rabbit complement added. Following further incubation for 45 min at 37 °C cells were counted with Trypan blue staining and inoculated to syngeneic A or (A $\times$ X) F₁ hybrids.

†The immunisation schedule was the same as for antiserum production.

‡A cytotoxic index was calculated by subtraction of the percentage of negative cells in the serum-treated sample from the percentage of negative cells in the control samples divided by the latter figure. The percentage of negatives in control serum ranged from 80 to 100%.

induced mouse lymphoma (YAC) and shown, in addition, that antigen(s) identical with or related to p15 and p12 are also expressed on the outer cell surface. Since these specificities were all detected with antisera produced in foreign species, the question as to which, if any of them, is responsible for MCSA, the antigen recognised by the syngeneic mouse—as mentioned above—remains open. We have approached this question by exposing the YAC lymphoma repeatedly to the immunoselective pressure of anti-MCSA sera and passage through preimmunised syngeneic hosts, until all detectable MCSA has disappeared. The immuno-resistant sublines were compared with the original YAC line with regard to the expression of the various virion antigens. The results support that MCSA differs from the known virion antigens, partly or completely.

The combined *in vitro* and *in vivo* selection of the first immuno-resistant subline (YACIR) was reported earlier¹⁷. Selection involved repeated exposure to mouse anti-MCSA sera in the presence of complement, before each passage *in vivo*. The sera were produced in A $\times$ C57BL or A $\times$

C57L F₁ mice, 3-6 immunisations with 6,000 r. irradiated YAC cells. After each cytotoxic exposure, surviving cells were passaged in syngeneic or semisyngeneic (A F₁) hosts, preimmunised with one inoculation of 6,000 r. irradiated YAC cells 2-2 weeks earlier. After ten such passages, the YACIR subline was completely resistant to the cytotoxic effect of the MCSA antiserum. Quantitative absorption experiments showed that MCSA was still present, but its concentration had decreased tenfold.

For the present study, we have repeated the immunoselection experiment and obtained a second, independent immuno-resistant subline (Table 1). Designated YAC 462-IR, it was completely resistant against the cytotoxic effect of the standard anti-MCSA serum.

For the cytotoxic tests, we used a microassay in which the serial serum dilutions in 0.002-ml volumes were injected under paraffin oil into rings of microtrays (Møller-Coats A/S, Norway) with an automatic Hamilton syringe. Cells (10^3 in 0.001 ml) were injected into the antiserum drop and the plates incubated for 20 min at 37 °C. Subsequently, 0.001 ml rabbit complement was added. The final dilution of complement was 1:40 for YAC, RBL-5 and FLC, 1:20 for YACIR, 3T3 and JLS-V9 cells, 1:8 for YAC462-IR and 1:80 for A lymph node cells. Plates were incubated further for 45 min. Thereafter 0.0005 ml Trypan blue was injected into the same drop and the results read under an inverted microscope.

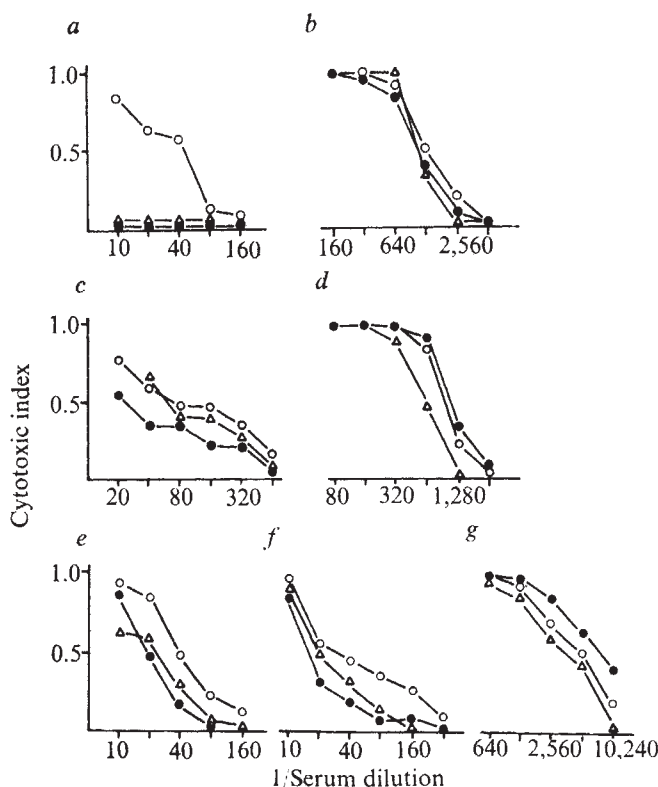
The goat anti-RLV (Rauscher leukaemia virus) sera were kindly provided by: Dr D. C. Fish, (anti-p30); Dr J. R. Stephenson (anti-p12) and Dr M. Strand (anti-gp69/71). The rabbit antisera against Friend virus proteins were kindly provided by Dr D. P. Bolognesi, (anti-p15) and Dr W. Schäfer (anti-gp71).

Figure 1a shows the high sensitivity of the original YAC line to anti-MCSA serum in sharp contrast against the complete resistance of the YACIR and YAC462-IR sublines. All three lines were equally sensitive to a rabbit anti-mouse serum (Fig. 1b) (raised by hyperimmunising a rabbit with mouse tumour cells induced by methylcholanthrene). Moreover, all lines were about equally sensitive to all tested reagents against the following purified virion components: p30, p12 and gp69/71 prepared from RLV (Fig. 1e-g); p15 and gp71 prepared from FV (Fig. 1c and d).

None of the antisera reacted with a series of control mouse cells, including normal A lymphocytes and the cell lines, NIH Swiss/3T3, BALB/3T3 and JLS-V9 (Fig. 2). The specificity of the anti-p30, anti-p15, anti-p12 and anti-gp71 sera was further confirmed by inhibition experiments with the corresponding purified protein (Table 2). These results suggest that MCSA is not identical with the p30, p15, p12 and gp71 specificities, expressed equally on the surface of YAC cells and the two IR-sublines.

It has been suggested that p30 is the principal cell surface antigen recognised by the rat¹³⁻¹⁵. Geering *et al.*¹⁶ have shown that the rat can recognise "group specific" antigens

Fig. 1 Cytotoxic sensitivity of YAC (○), YACIR (●) and YAC 462-IR cells (△) to the antisera as indicated. a, Mouse anti-MCSA; b, rabbit anti-mouse; c, rabbit anti-FVp15; d, rabbit anti-FVgp 71; e, goat anti-RLVp30; f, goat-RLVp12; g, goat anti-RLVgp69/71.



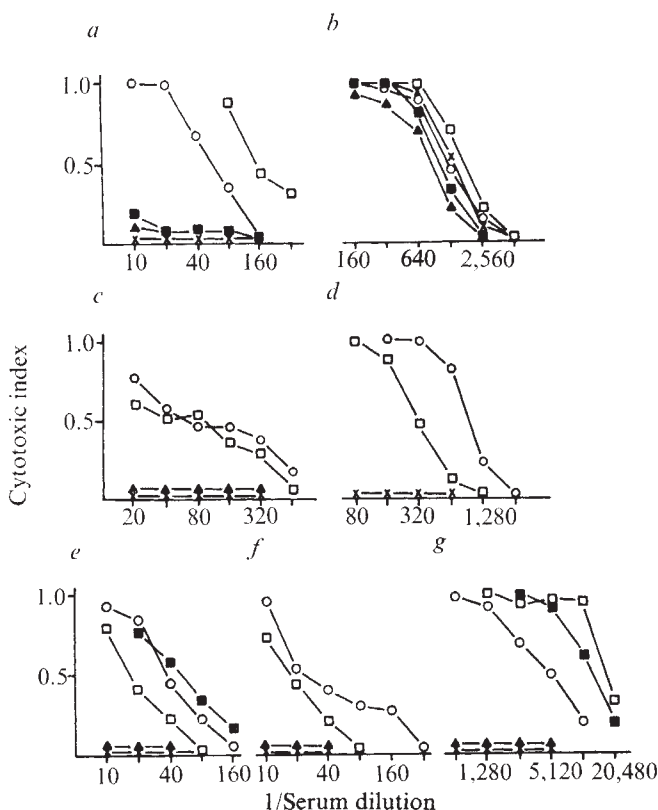


Fig. 2 Cytotoxic sensitivity of lymph node cells (×) of normal A mice, JLS-V9 cells (▲) and 3T3 cells (▲) of NIH Swiss and BALB/c origin, against YAC (○), RBL-5 (□) and F-765 (■). RBL-5 is an RLV-induced leukaemia cell line of C57BL origin and is grown *in vitro* as a suspension culture. F-765 was established *in vitro* as a suspension culture from the spleen of a DBA/2 mouse infected with FV. *a-g*, As for fig. 1.

(p30 in modern terminology) on the surface of leukaemia cells induced by mouse leukaemia virus, whereas the same antigen(s) remain unrecognised in the natural murine host¹⁷.

It was shown recently that p15 is present on the virion envelope¹⁸ and behaves mainly as an interspecies specific antigen. This antigen is recognised by the mouse and may even induce naturally occurring antibodies^{17,19}. Both MCSA and TATA are leukaemia virus strain specific, however, in contrast to what has been claimed for p15 and in line with our findings that dissociate p15 clearly from MCSA.

In conclusion, the present work shows that repeated immunoselection, alternating between *in vitro* cytotoxic treatment with anti-MCSA sera, in the presence of complement, and passage through preimmunised mice, leads to immunoresistant sublines. Such lines are resistant to anti-MCSA sera, but their sensitivity to the cytotoxic effect of antisera directed against p12, p15, p30 and gp69/71 virion

antigens remained unchanged. This suggests that the syngeneic murine host recognises a virally determined surface antigen, MCSA, that differs from known virion antigens partly or completely.

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Association of Epstein-Barr viral genomes with American Burkitt lymphoma

THE African form of Burkitt lymphoma (BL) is consistently associated with Epstein-Barr virus (EBV), as judged from the presence of elevated serum titres against viral antigens, and the occurrence of EBV DNA and EBV-associated nuclear antigen (EBNA) in the tumour cells^{1,2}. It has been postulated that EBV has a role in the causation of BL in Africa. It has also been suggested that aetiological cofactors contribute to the high tumour incidence in the endemic areas. In particular malaria has been proposed by Burkitt³ because of the geographical coincidence of BL and holo- or hyperendemic malaria. Rare cases of BL occur outside Africa but have not been shown to be associated with EBV⁴. We now present evidence for such an association.

We have studied 12 American patients with the diagnosis BL at the National Cancer Institute, for whom both sera and tumour tissue were available. Sera were assayed for antibody titres to several EBV-associated antigens. Tumour samples were transported frozen to Stockholm for assays of EBV DNA by nucleic acid hybridisation, using ³²P-labelled complementary RNA (cRNA) according to Lindahl *et al.*².

Among these 12 sera, two (R.A. and R.B.) had an antiviral capsid antibody (VCA) titre of $\geq 1:160$, whereas the other ten had lower titres (see Table 1). Antibodies to the diffuse type of the early antigen (EA(D)) were high in only one case (R.A.), the remaining patients having low EA(D) titres (see Table 1). Only one patient (R.B.) out of 11 had a measurable antibody titre (1:40) to the restricted (R) type of EA, frequent in African BL patients; but serum from R.A. could not be evaluated in this regard.

Table 1 shows that positive hybridisation results were obtained with EBV cRNA on two separate tumour specimens from one patient (R.A.). Multiple EBV genome copies were present, as in African BL biopsies². Another case (R.B.) was

Table 2 Inhibition of cytotoxicity of antisera with the corresponding virion protein

Serum	Protein	Amount needed for 100% inhibition (ng)	
		RBL-5	YAC
Goat anti-RLVp30	RLVp30	72	36
Goat anti-RLVp12	RLVp12	100	100
Rabbit anti-FVp15	FVp15	125	62.5
Rabbit anti-FVgp71	FVgp71	125	125

The inhibition assay was carried out in the microcytotoxicity assay. The proteins were diluted in antiserum, at dilutions giving 70–90% killing. Ten twofold dilution steps of the proteins in 2 or 3 dilutions of antiserum were included in each test. The cells and complement were added to the drops containing the antiserum-protein mixtures. None of the proteins (500 ng) inhibited the cytotoxic activity of a rabbit anti-mouse serum.

Table 1 Serum titres and nucleic acid hybridisation with EBV ³²P-cRNA*

Source of DNA	VCA	EA(D)	Serum titres EA(R)	EBNA	³² P cRNA bound per 10 µg DNA (c.p.m.†)	EBV genome equivalents per cell‡	No. of determinations
Calf thymus					218	0	6
Raji					4,860	60	6
R.A. bone marrow, (50% tumour cells)	320	160	?	160	1,014	10	6
R.A. lymph node (solid pieces)					3,158	38	4
R.A. lymph node (cells in suspension)					4,550	56	3
R.B. ascites	160	10	40	160	340	~ 2	4
R.K. ascites	10	< 10	< 10	20	221	< 1	2
S.V. ascites	10	< 10	< 10	2	229	< 1	2
P.R. pleural fluid	40	< 10	< 10	40	211	< 1	2
S.L. ascites	20	< 10	< 10	10	196	< 1	2
D.T. leukocytes (20% tumour cells)	80	< 10	< 10	2	229	< 1	2
S.L. leukocytes (90% tumour cells)	20	< 10	< 10	40	237	< 1	2
K.K. pleural fluid	< 10	< 10	< 10	< 2	201	< 1	2
R.L. testes	20	< 10	< 10	20	239	< 1	2
E.W. ovary	20	< 10	< 10	40	223	< 1	2
L.D. ascites	40	< 10	< 10	10	220	< 1	2

*Initial specific activity 1.2×10^8 c.p.m. μg^{-1} .

†DNA content on each filter after the hybridisation was determined by the diphenylamine reaction and used to correct the data to 10 μg DNA filter. For further details on the hybridisation procedure see ref. 2.

‡Number of EBV genome equivalents per cell were calculated with the assumption that the molecular weight of EBV DNA is 10^8 and that of cellular DNA 4×10^{12} .

borderline positive, because the data obtained were close to the limit of sensitivity of the hybridisation technique. The ascites specimen from this patient (R.B.) was EBNA positive (20% positive cells). As noted above, R.A. and R.B. were serologically unique in the present material, in having elevated EBV antibody titres and, in the case of R.B., even a clear EA(R) titre. Material from the other 10 tumour tissues gave negative results, that is, they could not have contained one EBV genome equivalent or more per cell.

The bone marrow aspirate of the positive RA patient contained approximately 50% tumour cells, and thus harboured about 20 EBV genome equivalents per tumour cell. Solid lymph node pieces contained at least 38 EBV genome equivalents per cell, whereas tumour cells in suspension contained about 56 EBV genome equivalents per cell. This patient was a white, 16-yr-old male with no record of malarial infection or travels to Africa. His clinical course was unusual in that he presented with cervical lymph node and bone marrow involvement several months after a syndrome compatible with acute infectious mononucleosis. Patient R.B. presented with large abdominal tumour and ascites; he died during induction therapy of acute pulmonary embolus.

Thus, in agreement with the results of Pagano *et al.*⁴ the majority of American patients with the diagnosis BL lack the serological or viral genome association with EBV documented in African BL biopsies. One clearly positive exception has been identified in this study and one probable (borderline) positive. In addition, a BL biopsy from Israel has been found to be EBNA positive and EBV DNA-positive (Goldblum *et al.*, unpublished). Similar data have been obtained independently in other laboratories. Bornkamm *et al.*⁵ have detected EBV DNA in a biopsy from a 6-yr-old German girl and Gravell *et al.*⁶ and Epstein *et al.*⁷ have each reported one case of an EBV DNA-carrying American BL patient. Thus, there is at present a total of five or six cases of non-endemic BL which harbour EBV in the tumour. The relatively large series reported here would suggest a rate of approximately 8–17% EBV-associated BL⁸ in the non-endemic material. Although the histopathology of the non-EBV-associated American BL cases resembles typical African BL, it is not clear whether these non-endemic cases represent the same disease entity or should be classified differently from a clinical or aetiological standpoint.

In the high endemic regions of Africa, BL is known to arise from a single EBV-carrying cell in more than 95% of cases^{9,13}. EBV-negative lymphomas classified as BL do not readily become infected with EBV *in vivo* even in cases in which the patients have been demonstrated to be carriers of non-malignant EBV-

transformed cells. One patient (K.K.) in the present series developed classical infectious mononucleosis while in remission; nevertheless, tumour cells collected at the time of EBV seroconversion (VCA 1:40) were EBV genome negative. Horizontal infection *in vivo* may be hampered by the presence of virus-neutralising antibodies, sensitised memory T cells, and/or the virus receptor status of the tumour. The latter varies from total resistance to *in vitro* infection, to relative susceptibility in cultured EBV-negative BL lines^{10–12}.

Since histology and cytology are the sole criteria for the diagnosis of BL at present, the high frequency of EBV-positive tumours in endemic regions, and their rare occurrence in non-endemic areas, may imply disease heterogeneity in spite of morphological uniformity. It has recently been proposed that EBV-associated BL and EBV-negative BL may be two different diseases with very similar histopathological features⁸, and there are abundant parallel examples of this in the experimental literature on known virus-induced tumours. If EBV is aetiologically important in EBV-associated BL, it would seem likely that one or more cofactors are involved which could be mediated by environmental (for example, malaria), genetic (for example, chromosomal), immunological (for example, impaired surveillance), viral or other mechanisms which exert a major influence in endemic regions, but a sporadic effect in non-endemic areas. This influence may not be identical in nature, but similar in action in these differing regions.

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Inhibition of the transition of a 40S ribosome–Met-tRNA_i^{Met} complex to an 80S ribosome–Met-tRNA_i^{Met} complex by 7-methylguanosine-5'-phosphate

7-METHYLGUANOSINE-5'-PHOSPHATE (m⁷G⁵p) inhibits *in vitro* amino acid incorporation catalysed by several eukaryotic mRNAs¹. The analogues, 7-methylguanosine-2(3)-phosphate and unmethylated guanosine-5'-phosphate have no effect on these reactions, suggesting that the specific inhibition by m⁷G⁵p is related to the m⁷G⁵ppp cap known to be present at the 5' end of many eukaryotic and viral mRNAs^{2–12}. As a more direct test of this idea, we report here experiments with a fractionated protein chain initiating system from wheat germ in which mRNA is required for the conversion of a 40S–Met-tRNA_i^{Met} complex to an 80S–Met-tRNA_i^{Met} complex. Using several viral RNAs, we show that m⁷G⁵p inhibits only the mRNA-dependent step, and that this inhibition is demonstrable only with mRNAs that require a 5' cap for translational activity.

The initiation process in the fractionated wheat system proceeds in three steps: formation of a factor–Met-tRNA_i^{Met} complex, transfer of the complex to a 40S ribosomal subunit, and adjunction of the 60S ribosomal subunit to form an 80S ribosome–Met-tRNA_i^{Met} complex. Figure 1a demonstrates the reaction leading to the formation of the 40S–Met-tRNA_i^{Met} complex. This reaction requires two factors and GTP, and is unaffected by addition of mRNA. Figure 1b shows that further incubation at 3.6 mM Mg²⁺ with added ATP, mRNA and a third factor results in the formation of an

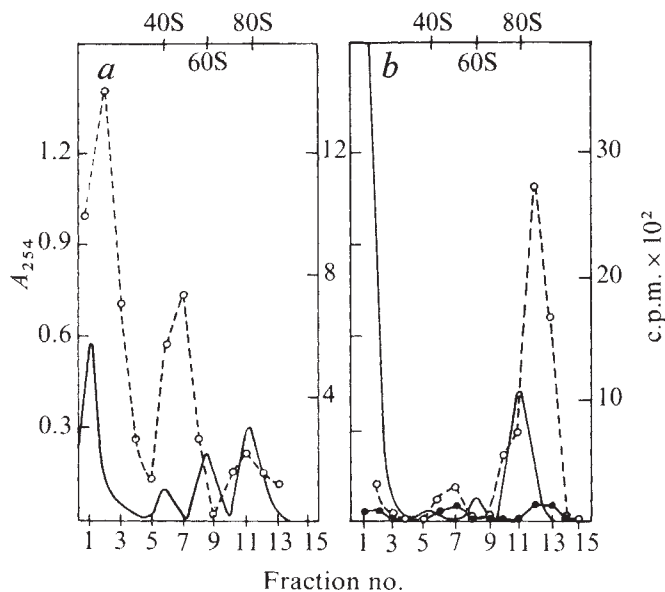


Fig. 1 Sequential formation of 40S–Met-tRNA_i^{Met} and 80S–Met-tRNA_i^{Met} ribosome complexes. *a*, 40S reaction. A reaction mixture (0.34 ml) containing 1 mM unlabelled Met; 30 mM Tris acetate, pH 8.0; 2.6 mM dithiothreitol; 60 μM GTP, 70 mM K acetate; 5 mM KCl; 1.3 mM Mg acetate; 7 mM phosphoenolpyruvate; 11.2 μg pyruvate kinase; 39 μg tRNA containing 6.6 pmol (CH₃-³H)Met-tRNA_i^{Met} (2,300 c.p.m. pmol⁻¹); 25 μg factor C3β; 150 μg factor D, and 115 μg ribosomes was incubated for 10 min at 20 °C. The samples were fixed with glutaraldehyde, centrifuged through a 13–21% sucrose gradient, and the fractions were analysed for bound aminoacyl-tRNA by nitrocellulose filtration. *b*, 80S reaction: the reaction was carried out in two steps. A reaction mixture identical to that of (*a*) was first incubated for 2 min at 20 °C, then 35 μg factor C3α, 6 μg AMV RNA, ATP (1 mM), Mg acetate (3.6 mM), and K acetate (70 mM) were added to a volume of 0.40 ml and the incubation was continued for 10 min at 20 °C. The samples were centrifuged without glutaraldehyde fixation through sucrose gradients and analysed as in (*a*). The open circles denote the data with the complete reaction mixture, while the solid circles denote data for a reaction mixture in which mRNA was omitted. AMV RNA is the RNA of the top “a” component of alfalfa mosaic virus¹³ and was provided by Dr Lous Van Vloten-Doting. A complete description of the preparation of the wheat factors will be reported elsewhere.

Table 1 Effect of guanosine monophosphates on the formation of 40S–Met-tRNA_i^{Met} and 80S–Met-tRNA_i^{Met} complexes

	Addition	Met-tRNA _i ^{Met} bound (c.p.m.)	Inhibition (%)
(1) 40S reaction	—	1,701	
	m ⁷ G ⁵ p	1,640	3.6
	m ⁷ G ²⁽³⁾ p	1,829	
	G ⁵ p	1,876	
(2) 80S reaction (TMV RNA)	—	1,706	
	m ⁷ G ⁵ p	342	80.0
	m ⁷ G ²⁽³⁾ p	1,468	14.0
	G ⁵ p	1,397	18.1
(3) 80S reaction (STNV RNA)	—	1,953	
	m ⁷ G ⁵ p	2,147	
(4) 80S reaction (AMV RNA)	—	5,297	
	m ⁷ G ⁵ p	1,363	74.3

The reaction systems were those of Fig. 1, with the viral RNAs added at 6 μg per 0.34 ml total volume. The data are a calculated sum of the nitrocellulose bound radioactivity; in experiment 1 for the region of the 40S complex and experiments 2–4 for the region of the 80S complex. The data in experiments 2–4 are corrected for a control in which mRNA was omitted. Procedures for preparation of TMV RNA and STNV RNA are described in refs 14 and 15, respectively. m⁷G⁵p and G⁵p (PL Biochemicals) and m⁷G²⁽³⁾p (TerraMarine Bioresearch) were added as indicated at a concentration of 0.25 mM.

80S–Met-tRNA_i^{Met} complex. The effect of several guanosine monophosphates on these reactions is examined in Table 1. Experiment 1 shows that the 40S reaction is unaffected in all cases. In the 80S reaction, m⁷G⁵p is strongly inhibitory, while the other guanosine mononucleotides have only a small effect (experiment 2), a specificity similar to that observed for the inhibition of overall amino acid incorporation¹. The localisation of the inhibition by m⁷G⁵p to the reaction in which the 80S–Met-tRNA_i^{Met} is formed, that is, the sequence in which mRNA participates, supports the idea that m⁷G⁵p inhibition is related to its structural analogy with the 5'-m⁷G⁵ppp present on most eukaryotic mRNAs.

As a further test of this conclusion, we studied the effect of m⁷G⁵p on the 40S–Met-tRNA_i^{Met} to 80S–Met-tRNA_i^{Met} reaction catalysed by RNA of satellite tobacco necrosis virus (STNV RNA). This RNA has been reported to have a 5'-terminal sequence of either ppApGpUp...¹⁶ or pppApGpUp...¹⁷ and we have further confirmed that it lacks an m⁷G⁵p terminus by examining the products of HIO₄ oxidation and ³H-borohydride reduction (unpublished experiments with S. Muthukrishnan and A. Shatkin). We have also ascertained that STNV RNA does not require a m⁷G⁵ppp cap for its translation. Translation of STNV RNA is unaffected, either quantitatively (Table 2) or qualitatively (Fig. 2), by addition of *S*-adenosylhomocysteine (SAH), a methyl-transfer inhibitor known to prevent the ‘capping’ reaction¹⁸. Thus STNV RNA is ideal for testing whether the inhibitory action of m⁷G⁵p

is related to its structural similarity to the $m^7G^{5'}ppp$ 'cap' of an mRNA. If it were, one would predict that $m^7G^{5'}p$ would have little or no effect on the reaction catalysed by STNV RNA. Experiment 3 of Table 1 verifies this prediction. STNV RNA-catalysed conversion of the 40S-Met-tRNA_i^{Met} complex to an 80S-Met-tRNA_i^{Met} complex is unaffected by $m^7G^{5'}p$. Viewed in contrast to the results with tobacco mosaic virus (TMV RNA) (experiment 2) and alfalfa mosaic virus (AMV RNA) (experiment 4), both of which contain the 5' 'cap'¹⁰⁻¹², the action of $m^7G^{5'}p$ indeed seems to be related to its structural analogy to the $m^7G^{5'}ppp$ 'cap' of mRNA.

The maximal inhibition that we have been able to obtain with either TMV RNA or AMV RNA is 80% even at $m^7G^{5'}p$ concentrations as high as 1 mM. With TMV RNA, it has been reported that only 70% of the viral RNA population contains the $m^7G^{5'}ppp$ 'cap'^{10,11}. Thus the incomplete inhibition by $m^7G^{5'}p$ might be ascribed to residual activity of those RNAs lacking the 'cap'. We favour, however, an alternative explanation; that the translational requirement for the $m^7G^{5'}ppp$ 'cap' is not absolute even for those mRNAs containing the 5' 'cap' but rather that the $m^7G^{5'}ppp$ 'cap' provides an additional binding site in the initiation system, thereby augmenting the translation of a particular mRNA. This possibility would allow that mRNAs might differ widely in the translational advantage gained by the 'cap', with the extreme situation being that exemplified by STNV RNA, where translation occurs readily in the absence of the 5'- $m^7G^{5'}ppp$.

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Table 2 ¹⁴C-leucine incorporation catalysed by STNV RNA in presence and absence of SAH

Additions	¹⁴ C-leucine incorporated (c.p.m.)
—	1,200
STNV RNA (2.5 µg)	24,331
STNV RNA + SAM (4 µM)	26,752
STNV RNA + SAH (320 µM)	25,250
reovirus RNA (2.5 µg)	8,873
reovirus RNA + SAM (4 µM)	13,944
reovirus RNA + SAH (320 µM)	2,480

A 0.1-ml incubation mixture containing 20 mM HEPES-KOH, pH 7.6, 1 mM ATP, 20 µM GTP, 8 mM creatine phosphate, creatine phosphokinase (40 µg ml⁻¹), 2.5 mM dithiothreitol, 2 mM Mg acetate, 80 µM spermine, 20 mM KCl, 120 mM K acetate, 30 µM 19 amino acids-leucine, ¹⁴C-L-leucine (0.625 µCi ml⁻¹), wheat tRNA (15 µg ml⁻¹), 25 µl wheat germ S23, and viral RNA as indicated, was incubated for 60 min at 25 °C. The hot TCA-insoluble radioactivity was determined as before¹⁴. Wheat germ S23 was prepared by a modification of the earlier procedure developed for wheat embryo¹⁹. A sample of 1.5 g of commercial wheat germ (General Mills) was ground in 10 ml 1 mM Mg acetate, 2 mM CaCl₂, 90 mM KCl using a chilled mortar and pestle. The homogenate was centrifuged at 23,000g for 10 min (pellet discarded), adjusted to 20 mM Tris acetate, pH 7.6, 2 mM Mg acetate and recentrifuged as above. The supernatant was then passed through a 25 × 1.5 cm column of Sephadex G-25 and eluted with 1 mM Tris acetate, pH 7.6, 50 mM KCl, 1 mM Mg acetate, 4 mM β-mercaptoethanol. The turbid fraction was collected, centrifuged at 23,000g for 10 min (pellet discarded), and stored in small samples at -70 °C. Reovirus RNA with a 5'-terminus of 75% ppGp and 25% GpppG was a gift of M. Morgan and A. Shatkin. S-adenosylmethionine (SAM) and S-adenosyl homocysteine (SAH) were obtained from Sigma.

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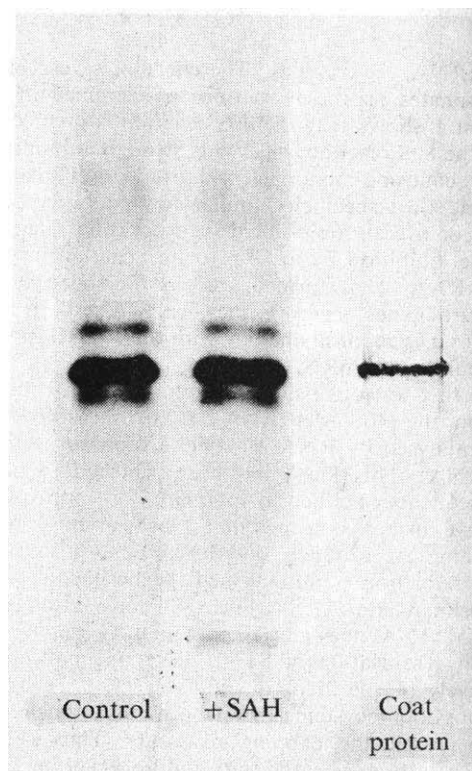
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Fig. 2 *In vitro* products synthesised in response to STNV RNA. The *in vitro* incorporation reaction was carried out as in Table 2 except that a 19-methionine amino acid mixture and 20 µCi ³⁵S-L-methionine were used in place of leucine. The reaction was terminated by the addition of 10 mM Na₂-EDTA, pH 7.0, 10 mM L-methionine, and ribonuclease (0.1 mg ml⁻¹). After 15 min at 25 °C the mixture was cooled and precipitated with 9 volumes of cold acetone. The precipitated protein was air dried, dissolved in 4 M urea, 2.5 mM dithiothreitol, 1% sodium dodecyl sulphate, 50 mM Tris-HCl, pH 6.8, 1 mM Na₂-EDTA, pH 7.0, and heated at 100 °C for 2 min. Aliquots of the sample were applied to 15% polyacrylamide-urea-SDS slab gels prepared according to Studier²⁰, and electrophoresed at 80 V for 5-6 h. The gel was stained with 0.75% Coomassie blue, destained with H₂O-methanol-glacial acetic acid (7:4:1), dried and autoradiographed. Authentic STNV coat protein was prepared according to Lesnaw and Reichmann²¹.



Structure of the lactose operator

THE *lac* operon has long been an important model system for developing an understanding of the control of gene expression. Since the early studies of Jacob and Monod¹ on the *lac* operon, much work has been devoted towards determining the mode of interaction between repressor and operator. Although it has been shown clearly that repressor physically binds operator^{2,3},

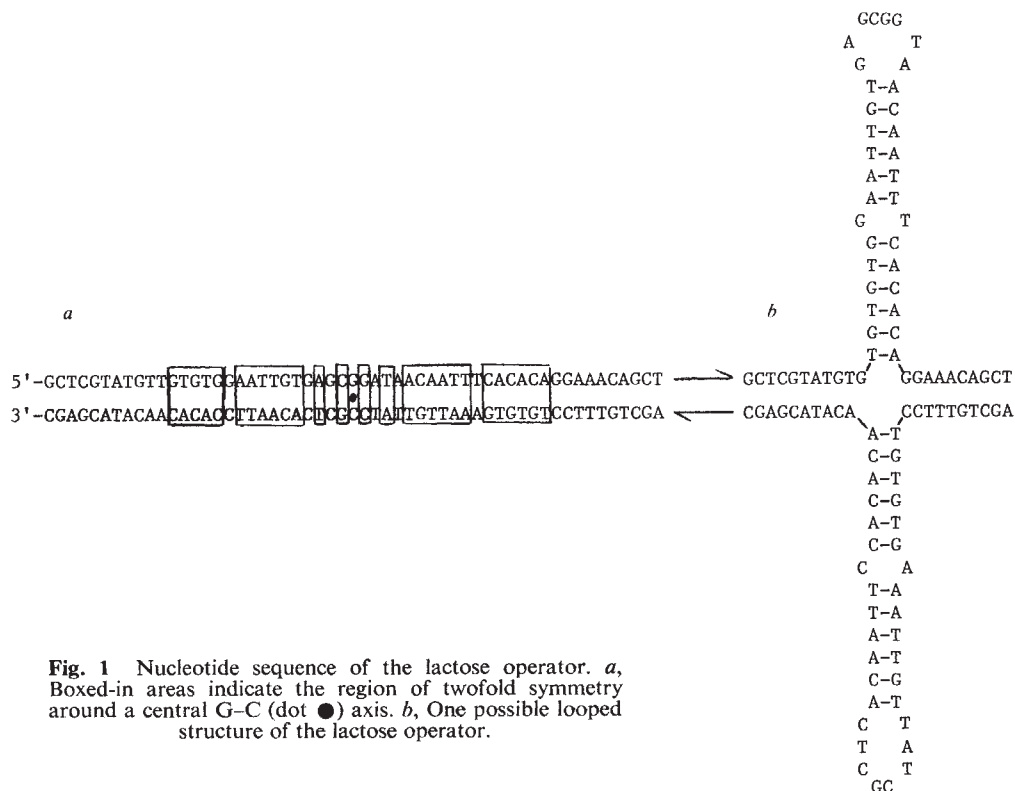


Fig. 1 Nucleotide sequence of the lactose operator. *a*, Boxed-in areas indicate the region of twofold symmetry around a central G-C (dot ●) axis. *b*, One possible looped structure of the lactose operator.

the details of this interaction remain unknown. We report here results of experiments designed to investigate further the structure of the operator and its interaction with the repressor.

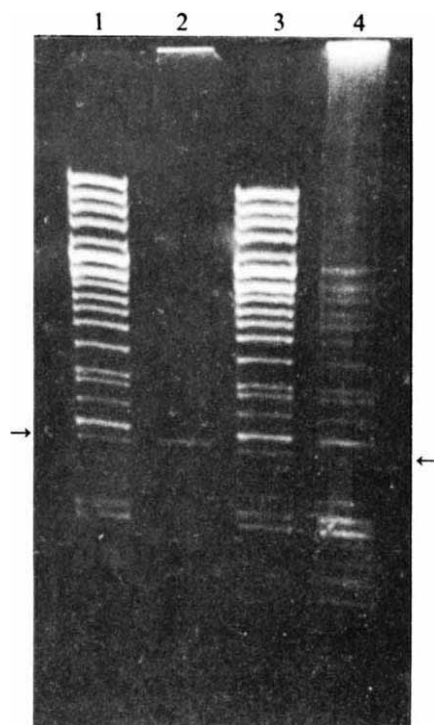
The interaction between *lac* operator and *lac* repressor is one of the most highly selective processes in nature. What is it about the *lac* operator, a sequence of approximately 25 nucleotides⁴, which allows *lac* repressor to select it from among one million bases in *E. coli*? Basically, when considering the mode of interaction between repressor and operator, theories fall into two categories: those which call for a major alteration such as single-stranded loop formation of the operator DNA, and those which do not. It has been proposed that twofold symmetry would be important in DNA-protein interactions⁵, that regulatory proteins would bind to and stabilise single-stranded loop structures in DNA⁶, and that formation of a loop structure would allow a protein with two or four subunits to interact with symmetrical DNA sequences⁷.

The determination of the nucleotide sequence of the *lac* operator^{4,8} (Fig. 1) served to amplify all the above questions. The *lac* operator does show twofold symmetry, in fact the type of symmetry within the *lac* operator, termed a complementary palindrome⁹, theoretically allows the *lac* operator to form a looped structure (Fig. 1). We have tried to determine whether such a looped structure is indeed present, by using single-strand specific nucleases as probes.

We have examined the actions of two single-strand specific nucleases, S1 and mung bean, on a DNA fragment containing part of the *lac* operon (see Fig. 2 for identification). The fragment, produced from λ plac 5 DNA by digestion with the *Hind* restriction enzyme is approximately 700 base pairs long^{10,11} and contains the carboxyl terminal sequence of the *i* gene, the entire *lac* control region and about 30% of the *z* gene. The *lac* operator region is located almost in the centre of the fragment.

Figure 3 shows the results of control experiments carried out by incubating heat-denatured ³H-salmon sperm DNA with S1 nuclease (Fig. 3a) or mung bean nuclease (Fig. 3b). In 15 min, S1 nuclease at a concentration of only 0.1 U per μ g DNA rendered 50% of the single-stranded DNA acid soluble, and mung bean nuclease at a concentration of only 0.0065 U per μ g DNA rendered 80% acid soluble. Thus, at the much higher concentrations of S1 nuclease and mung bean nuclease used in

Fig. 2 λ plac 5 DNA (250 μ g; prepared from strain G141 (λ C₈₅₇ λ plac5), a gift from Dr W. Gilbert, by the method of ref. 18) was digested with the *Hind* restriction enzyme (prepared as in ref. 19 and a gift from Dr E. Jay). After phenol extraction and ethanol precipitation this digest was mixed with 1.5 μ g of *lac* repressor (prepared as in ref. 20) and passed through a Millipore HAWP, 0.45- μ m filter. The filter-bound material was eluted with 1 mM IPTG. All samples were precipitated in ethanol before application to a 20 cm $\times$ 20 cm $\times$ 0.3 cm slab gel of 1.4% Agarose (Seakem) and run in the presence of ethidium bromide. Electrophoresis was run at 80 V for 6 h. Slots 1 and 3 show the total *Hind* digest of λ plac5 DNA. Slot 2 shows the fragment eluted from the filter with IPTG. Slot 4 shows the filtrate. The arrows indicate the region of interest. Note that in slot 2 there is only one fragment which is bound specifically by *lac* repressor and can be eluted from the filter by IPTG. This fragment is absent in the filtrate (slot 4).



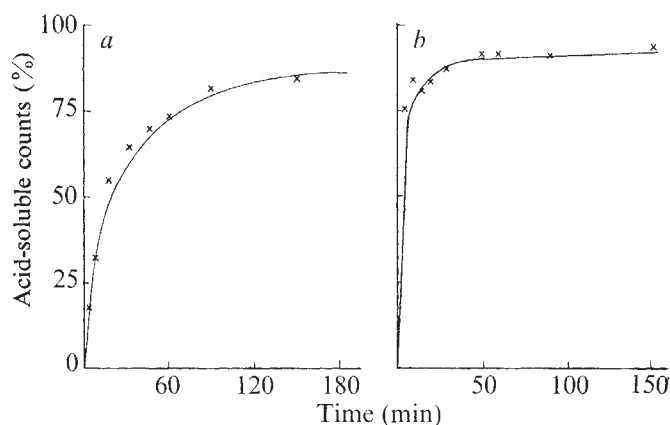


Fig. 3 Heat-denatured ^3H -salmon sperm DNA²¹ was incubated with: *a*, S1 nuclease at 0.1 unit per μg DNA at 37 °C; and *b*, mung bean nuclease at 0.0065 unit per μg DNA at 37 °C. S1 nuclease was prepared by the procedure of Vogt¹⁷ and was a gift of Dr G. Ghanges; the unit is as defined by Vogt¹⁷. Mung bean nuclease was a gift of Dr S. Laskowski, Sr, and the unit is as defined in Ardelt and Laskowski²². Incubations are as in the legend to Fig. 4. Each time point is the result of a triplicate determination.

the experiments below, single-stranded DNA would be hydrolysed to acid solubility in a few seconds.

The action of S1 and mung bean nuclease on the fragment is shown in Fig. 4. The incubation mixtures were analysed on $20 \times 20 \times 0.3$ cm slab gels of either 1.4% Agarose or 4 or 5% acrylamide-7 M urea. Figure 4*a* shows an Agarose gel of *lac* DNA fragment after treatment with various concentrations of S1 nuclease. No specific cleavage of the fragment can be detected, even at high concentrations of the nuclease. Since *lac* operator lies almost in the centre of the *Hind*-700 DNA fragment (arrow *A*), specific cleavage by S1 nuclease should yield two fragments about 350 base pairs long which would be clearly resolved (arrow *B*, expected location) from the starting material in the gel system. Analysis on acrylamide-7 M urea gels, which has a greater power of resolution, also failed to reveal any specific cleavage. Figure 4*b* shows the result of treating *Hind*-700 *lac* DNA fragment with high concentrations of S1 nuclease as analysed on

4% acrylamide-7 M urea. S1 nuclease incubations were normally done at 50 mM NaCl, and varying the NaCl concentration at low S1 nuclease concentrations did not change the patterns shown. Results with mung bean nuclease were essentially identical to those with S1 nuclease. Even at the highest enzyme concentrations used, no specific cleavage of the fragment could be detected on either 1.4% Agarose (Fig. 4*a*) or 4% acrylamide-7 M urea gel (Fig. 4*b*). Fragment reisolated from the gels after nuclease treatment can still bind *lac* repressor with nearly the same efficiency as before. In some parallel experiments involving *lac* DNA fragment and S1 nuclease, a small amount of single-stranded ^3H -salmon sperm DNA was included in the reaction mixture. An aliquot was taken, after S1 nuclease treatment, to determine the extent of acid solubility. In all cases the single-stranded DNA was rendered at least 70% acid soluble.

Some streaking of the DNA band on gel and a reduction in the intensity of the starting material was observed when very high concentrations of the enzyme or low concentrations of salt were used. This may be due to either a nonspecific degradation of the *lac* DNA fragment by a contaminant nuclease or nonspecific degradation by S1 nuclease of the DNA fragment at areas where "breathing" occurs. That this degradation is nonspecific and not due to a single-stranded looped structure in the *lac* operator was demonstrated by treating a control DNA fragment that does not contain the *lac* operon with the same concentrations of S1 nuclease or mung bean nuclease. Gel patterns identical to those given in Fig. 4*b* were found.

If the *lac* operator existed as a single-stranded structure single-strand specific nucleases would be expected to cleave the *Hind* fragment carrying the *lac* operon into two pieces of about equal size. We did not find this to be the case and conclude, therefore, that in the absence of repressor, the *lac* operator does not contain single-stranded regions to any appreciable extent. The same conclusion was reached by Wang *et al.*¹², who measured the changes in superhelicity of *lac* DNA on binding of repressor, and also by Richmond and Steitz¹³ who studied the binding of *lac* repressor to poly d(A)-d(T) fragments and suggested that the repressor binds to native DNA. On the other hand, Chan and Wells¹⁴ have shown by competition binding assays that S1 nuclease- or mung bean nuclease-treated λplac5 DNA loses the ability to bind *lac* repressor. This result implies

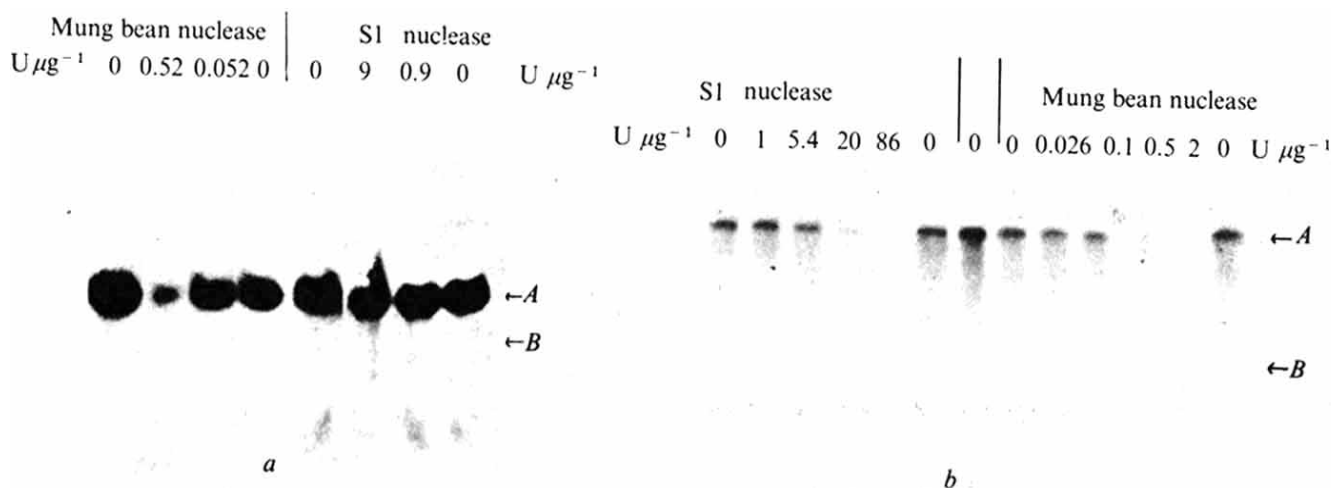


Fig. 4 *lac* fragment, identified as in Fig. 2 and purified as described²³, was labelled terminally at the 3' end with $\alpha\text{-}^{32}\text{P}$ -GTP or CTP using terminal transferase as described by Kossel and Roychoudhury²⁴. The fragment was then treated with a nuclease as follows. S1 nuclease: 30 mM NaOAc, pH 4.6, 1 mM ZnSO_4 , 5% glycerol, NaCl varied between 10 and 150 mM in 100 μl . DNA concentration was from 1 to 10 $\mu\text{g ml}^{-1}$. Mung bean nuclease: 25 mM NaOAc, pH 5.0, 1 mM 2-mercaptoethanol, 10 μM ZnSO_4 and 0.001% Triton X-100 in 100 μl . DNA concentration was from 0.1 to 1 $\mu\text{g ml}^{-1}$. Digestions were stopped by the addition of EDTA to 25 mM. Protein was removed by phenol extraction (twice). The DNA was then ethanol precipitated. The samples were dissolved in 20 μl of loading dye (*a*, 10 mM Tris-Cl, pH 7.9, 1 mM EDTA, 10% sucrose, 0.2 mg ml^{-1} bromophenol blue; *b*, as in *a* plus 7 M urea). Electrophoresis is from top to bottom on $20 \times 20 \times 0.3$ cm vertical slab gel apparatus. The wet gels were autoradiographed. *a*, *lac* fragment treated with S1 and mung bean nucleases at the indicated concentrations and then electrophoresed (80 V, 3.5 h) on 1.4% Agarose gel. *b*, *lac* fragment treated with S1 and mung bean nucleases at the indicated concentrations were boiled in loading dye plus 7 M urea for 5 min before electrophoresis on 4% acrylamide-7 M urea at 80 V for 24 h.

that S1 nuclease is cutting at or near the *lac* operator since binding of *lac* repressor before nuclease treatment abolishes the effect. Our experiments clearly demonstrate that a specific cleavage by a single-strand specific nuclease is not occurring within the *lac* operator region.

The nuclease concentrations used in our experiments are extremely high compared with those needed to hydrolyse single-stranded nucleic acid. Rushizky *et al.*¹⁵ have shown that 0.0017 U of S1 nuclease per μg of MS2 RNA hydrolysed the RNA to acid solubility in 3 h at 23 °C. In addition, Shenk *et al.*¹⁶ have cleaved linear SV40 DNA, nicked in only one position, into two intact and distinct double-stranded pieces with S1 nuclease at a concentration equivalent to 90 U (of Vogt¹⁷) per μg DNA at 25 °C in 30 min. They were also able to cleave DNA containing one mismatched base pair with the same concentration of nuclease.

We have been able to duplicate (qualitatively but not quantitatively) the results of Chan and Wells¹⁴ in intact λ plac5 DNA using competition binding as an assay, however, the same experiments analysed by electrophoresis in 0.4% Agarose gel showed no specific cleavage of the DNA. We therefore conclude that Chan and Wells may be observing a secondary or indirect effect of the nuclease which, perhaps by nicking certain A-T-rich regions of the DNA, somehow alters the repressor binding of the *lac* operator region.

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Cycling of RNA-directed DNA polymerase on natural and synthetic RNA templates

RNA-DIRECTED DNA polymerase (reverse transcriptase) catalyses *in vivo* the synthesis of DNA complementary to the RNA of tumour viruses. The DNA is integrated into the host genome and is therefore implicated in the subsequent tumorigenesis¹. The mechanism by which reverse transcriptase replicates the viral RNA into complementary DNA (cDNA) and its resultant integration, as well as the interaction of the viral components in the host, remain unclear². Reverse transcriptase has been used *in vitro* to synthesise cDNA to mRNA;

this cDNA can be used to identify and characterise the cellular processes dependent on nucleic acids. The conditions of the reactions have been optimised^{3,4}; however problems remain with both the quality and quantity of the cDNA^{5,6,15}. We report here *in vitro* conditions such that each RNA-directed DNA polymerase molecule synthesises several to more than 100 transcripts, depending on whether natural or synthetic templates are used. As noted previously^{3,4}, the fidelity of the cDNA transcript, the amount, and the time course of polymerisation depend strongly on the reaction conditions.

Highly purified DNA polymerase from avian myeloblastosis virus (AMV) strain BAL was prepared by the method of Kacian and Spiegelman⁷. Purification included collection of the virus from plasma by high speed centrifugation, batch elution from DEAE-cellulose and chromatography on phosphocellulose. The enzyme was concentrated by dialysis against: 50% glycerol; 0.2 M KPO₄, pH 7.2; 2 mM dithiothreitol and 0.2% Triton X-100. This preparation (No. XL 13-75) contained 61 μg of protein per ml and had an activity of 2,476 U ml⁻¹. The protein concentration was determined by the method of Lowry.

Examination of this preparation of DNA polymerase by sodium dodecyl sulphate (SDS) polyacrylamide gels⁸ demonstrated three distinct bands corresponding to the α and β subunits of the DNA polymerase and a contaminating band corresponding to a polypeptide chain of molecular weight 80,000. Based on the relative intensities of the stained bands the enzyme preparation was approximately 80% pure.

The reaction conditions are modified from those previously described⁹ and are optimised for the reinitiation and elongation of the cDNA. The increased purity of the enzyme preparation facilitated the establishment of conditions for the maximum synthesis of cDNA per unit of enzyme (Table 1). The optimal

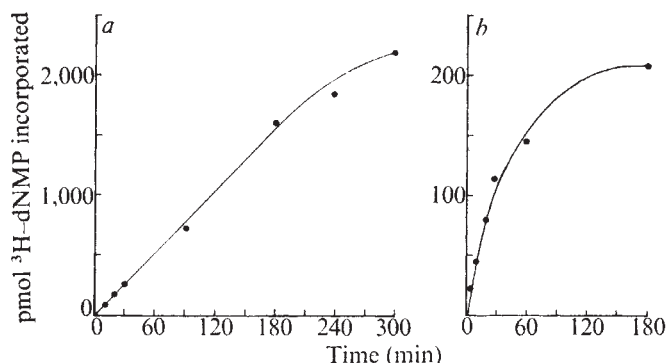


Fig. 1 Time course of cDNA synthesis as directed by poly(C) and rabbit reticulocyte globin mRNA. *a*, Time course of dGMP incorporation into cDNA directed by poly(C). The amounts of template, primer and radioactive nucleotide per ml were 570 μg of poly(C), 460 μg of oligo(dG)₁₀, and 25 μCi of 8-³H(N)-deoxyguanosine 5'-triphosphate, specific activity 9.4 Ci mmol⁻¹ (New England Nuclear). *b*, Time course of dCMP polymerisation into cDNA directed by globin mRNA template. The concentrations of primer, template and radioactive nucleotide per ml were 54 μg of globin mRNA, 24 μg of oligo(dT)₁₀ and 25 μCi of 5-³H-deoxycytosine 5'-triphosphate, specific activity 22.8 Ci mmol⁻¹ (New England Nuclear) or CH₃-³H-deoxythymidine 5'-triphosphate (specific activity 49.8 Ci mmol⁻¹, New England Nuclear). Oligoguanilic acid and oligothymidilic acid primers were obtained from Collaborative Research; poly(C) template with an S_{20,w} value of 3.08 was obtained from P.L. Biochemicals, and rabbit reticulocyte 10S mRNA was prepared as before¹⁰. Each 50 μl of reaction mixture contained the following components: 50 mM Tris-HCl, pH 7.9; 100 mM KCl, 10 mM MgCl₂; 8 mM dithiothreitol, actinomycin D (100 μg ml⁻¹) and AMV DNA polymerase (30 U ml⁻¹). The substrates were 320 μmol of each of the four deoxynucleoside triphosphates. The reaction mixtures were incubated at 32 °C for the times indicated. The reaction was terminated by the addition of ice-cold 10% trichloroacetic acid (TCA)–2.0% sodium pyrophosphate solution. After 30 min on ice, the TCA-precipitable material was collected by filtration on to prewashed (5% TCA–2.0% pyrophosphate) nitrocellulose filters. (Millipore type HA). The filters were then washed with 20 ml of TCA pyrophosphate, dried and the radioactivity determined by liquid scintillation spectroscopy.

Table 1 Effect of AMV DNA polymerase concentration on globin cDNA synthesis

Enzyme (pmol)	Globin mRNA (pmol) Enzyme (pmol)	dCMP incorporated (pmol)	Product* (pmol)	Product (pmol) Enzyme (pmol)
1.96	6.6	361	3.21	1.7
0.95	13	313	2.78	2.9
0.48	26	230	2.03	4.3
0.24	54	148	1.32	5.7
0.12	104	66	0.59	4.9

Reactions were run for 20 min as described in the legend to Fig. 1b.

* Calculation of cDNA synthesised is discussed in the text.

concentration of DNA polymerase was 0.24 pmol per 50 μ l of reaction mixture. Although the turnover number (cDNA molecules synthesised per unit of enzyme per incubation) was maximised, the population of cDNA molecules synthesised was heterogeneous. We find that the ratio of DNA polymerase to template concentration affects both the quality and quantity of the cDNA polymerised. In the "slippage" reaction of the homopolymers (A) and (I), (which depend on multiple initiations) synthesis initiated with 400 ng of DNA polymerase synthesised an homogeneous product⁹; but polymerisation at 40 ng of enzyme results in a heterodisperse size distribution of the cDNA. This has also been observed for the globin mRNA-directed synthesis, although the shift in the size distribution was not as marked.

The ability to follow cDNA synthesis at prolonged time intervals (probably due to reduced RNase activity in the enzyme preparation) is another improvement. The time course of globin mRNA-directed dCMP synthesis was compared with dGMP incorporation directed by poly(C) (Fig. 1). Incorporation differed in that the globin mRNA-directed synthesis of cDNA remained linear for only 20 min compared with more than 180 min for poly(C)-directed polymerisation. The homopolymer poly(A) or poly(I)-directed synthesis of a homopolymer cDNA was also linear for more than 180 min. The decrease in linearity observed with globin mRNA was not caused by template degradation as no stimulation was found when further mRNA was added (unpublished).

The comparison of the different cDNA products polymerised with globin mRNA, poly(C), poly(A) and poly(I) as templates is shown in Table 2. Poly(C)-directed incorporation of dGMP yielded 1,601 pmol and since the average template size was 55 nucleotides, 29 pmol of full sized product were formed. Dividing the moles of product formed by the moles of enzyme input, more than 100 cDNA molecules were formed per enzyme molecule. Repeating this calculation for globin mRNA as template the turnover number is 7, assuming the mRNA to be 650 nucleotides long¹¹ and composed of 17.3% GMP¹². Synthesis directed by poly(A)₂₅ or by poly(I)₃₅ results in a product size larger than the starting material (16S to greater than 22S)⁹. The difficulty in estimating the ratio of enzyme molecules to product synthesised in a reaction which slips¹³ (that is, the product is larger than the template) is that the product size is heterodisperse. Poly(A) and poly(I) templates directed the incorporation of 827 pmol of dTMP and 573 pmol of dCMP,

respectively. Since these amounts are less than that incorporated into poly(C)-directed cDNA and since the products are much larger, the turnover numbers for the slippage reactions are small (≤ 1).

The calculation of turnover numbers assumes that the protein preparation is homogeneous and completely active. In this instance, a protein contaminant in the enzyme preparation was demonstrated. In the poly(C)-directed synthesis the primed portion of the cDNA is unlabelled and this may represent as much as 20% of the template molecule, and potentially more than 50% of the newly synthesised DNA. Finally, the product synthesised was assumed to be full sized. Although this seems to be true for the cDNA to poly(C) template (Fig. 2a), which

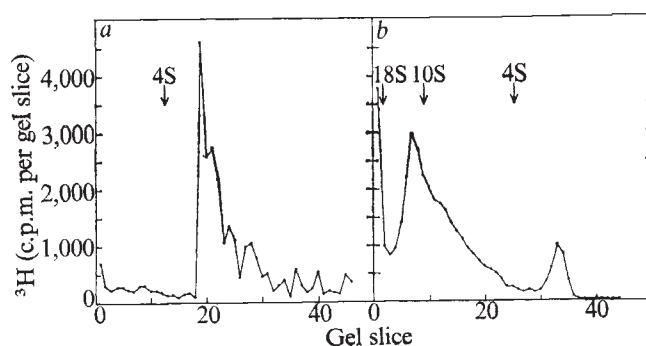


Fig. 2 Sizing of the cDNA product synthesised by DNA polymerase and directed by poly(C) and rabbit reticulocyte globin mRNA. Both reactions were carried out as described in the legend to Fig. 1 and incubated for 3 h. Poly(A) and poly(I) sizing was done as previously described⁹ and is not shown here. The reaction mixtures used to obtain cDNA product for size analysis by polyacrylamide gel electrophoresis were stopped by heating to 100 °C for 2 min and then quickly frozen on dry ice. The RNA was hydrolysed with 0.33 N NaOH at 37 °C for 18 h. After neutralising the hydrolysate, unreacted nucleotides were removed by dialysis at 4 °C against 0.5 M NH₄HCO₃ and finally distilled water. The lyophilised samples were resuspended and a 20- μ l sample was applied to 3.5% polyacrylamide gels containing sodium dodecylsulphate. The gels were standardised with rabbit reticulocyte 28S, 18S, 10S, and tRNA; poly(C), S_{20,w} 3.08. *a*, Size distribution of the cDNA product directed by poly(C); *b*, size distribution of the cDNA directed by globin mRNA (as discussed in the text).

Table 2 Comparison of AMV DNA polymerase-directed synthesis with different templates

Template	Primer	Size of template	dNMP incorporated (pmol)	Product (pmol)	Molecules cDNA per enzyme molecule
Globin mRNA	(dT) ₁₀	650	206*	1.8	7.2
Poly(C)	(dG) ₁₀	55	1,606	29.2	115
Poly(A)	(dT) ₁₀	25	827	?	?
Poly(I)	(dC) ₁₀	35	573	?	?

Reactions were run for 3 h as described in the legend to Fig. 1. All calculations correspond to a 50- μ l reaction volume. The size of the product was shown in Fig. 2a for the poly(C)-directed synthesis and Fig. 2b for the mRNA-directed synthesis. Slippage of poly(I) and (A) was described previously⁹ and confirmed by sodium dodecyl sulphate (SDS)-acrylamide gels (standardised with MS2 RNA and ϕ X174 DNA, Miles) as discussed in the legend to Fig. 2. Question marks indicate that product size was not calculated for these reactions because of the non-discrete product size, which occurs due to the slippage reaction with both poly(A) and poly(I) templates⁹.

* The quantity of dCMP incorporated into rabbit globin cDNA. Calculation of cDNA synthesised to globin mRNA is discussed in the text

showed an electrophoretic mobility faster than the 4S marker and corresponds to the template size, the mobility of globin cDNA was considerably more heterogeneous (as demonstrated in Fig. 2b). As the poly(C) or globin mRNA-directed product was no larger than its template, the turnover numbers listed in Table 2 must represent minimal values.

The size distribution of globin mRNA-directed cDNA is broader than the homopolymer-directed cDNAs (Fig. 2b). This distribution may represent either intrinsic heterogeneity of the template as demonstrated by Vournakis *et al.*¹⁴, or infidelity in the transcription reaction. No large (dT) homopolymers were detected and therefore there was no anomalous transcription of the poly(A) region as described by Falvey *et al.*¹³.

In summary, RNA-directed DNA polymerase initiates many template primer complexes and transcribes full sized cDNA from a template of either globin mRNA or poly(C). *In vitro*, the enzyme is amenable to Michaelis-Menton analysis since a stoichiometric reaction has been ruled out. Whether this ability to utilise more than a single template applies *in vivo* or whether reverse transcriptase is intrinsically controlled to polymerise only a single viral molecule awaits future study.

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Polymorphism of a synthetic DNA in solution

EARLY work on the X-ray diffraction of DNA fibres showed that DNA can adopt different conformations, and up to now at least four different double-helical models have been described in detail: the A, B, C, and D forms¹⁻⁴. During recent years, it has become likely that DNA in solution also occurs in different conformations⁵⁻⁷. For studying such helix-helix transitions synthetic polynucleotides with a defined sequence offer an important tool, since in natural DNA only a very small fraction of the molecule usually has a particular sequence and, sequence-specific effects of the conformation will therefore escape detection.

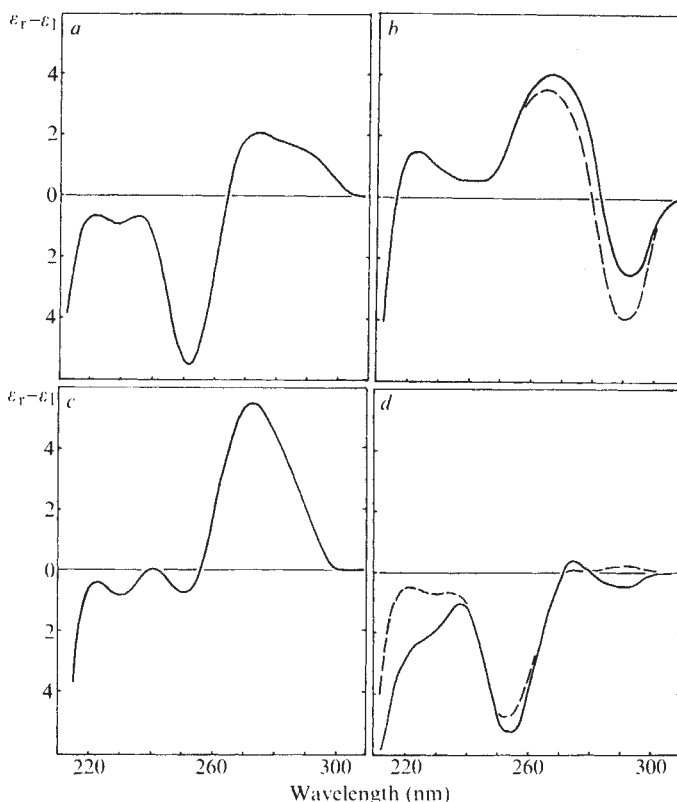
We have previously shown that the alternating desoxy-polynucleotide-poly(dG-dC)-poly(dG-dC)—undergoes a salt-induced, very cooperative helix-helix transition in neutral

solution, which is most easily followed by the inversion of the circular-dichroism spectrum in the near ultraviolet⁸. X-ray diffraction studies by S. Arnott on fibres of the same sample of poly(dG-dC) have led to diffraction patterns characteristic for the D form and, at high relative humidity, for the B form, respectively^{4,8} (personal communication). Since the fibres were pulled after ethanol precipitation of the polymers, it is possible that ethanol induces such a conformational change, which is then preserved in the fibres. I therefore measured the CD spectra of poly(dG-dC) as a function of the ethanol concentration. Figure 1 shows examples of CD spectra of poly(dG-dC)-poly(dG-dC) in different solvent conditions at room temperature. Between 0 and 45% ethanol the conservative spectrum observed is similar to that of natural DNA⁹ (Fig. 1a). At 50% ethanol a drastic and fully reversible change of the CD spectrum occurs, giving rise to a conservative, but inverted spectrum (Fig. 1b). The absorption and CD spectrum, the high cooperativity of the transition and the relative slow kinetics closely resemble the observations at high NaCl concentration in the absence of ethanol⁸. It is very likely that both solvents induce a change to the same double helical form.

At still higher ethanol concentrations a more gradual change to a non-conservative spectrum (Fig. 1c), typical for double-helical RNA-like conformations is observed^{5,6,10}. Figure 2 shows these changes of the optical properties as a function of the solvent composition. Thus, in ethanolic solution three distinct forms for poly(dG-dC) are observed.

A similar situation with three types of spectra exists in CsCl solutions in the absence of ethanol. Up to about 4 M CsCl the spectrum in Fig. 1a describes the data; around 4 M

Fig. 1 Circular dichroism spectra of poly(dG-dC)-poly(dG-dC) at different solvent composition. The polynucleotide was synthesised and characterised as described previously⁸. Ethanol was mixed with 1 mM Na phosphate buffer (pH 7.2) and a small volume of stock solution of poly(dG-dC) to a final concentration of 50-150 μ M was added, and the CD spectra measured at 20 °C in a Cary 60 spectropolarimeter with CD attachment. Absorption spectra were measured with a Zeiss PMQ 3 photometer. *a*, 0-40% (w/w) ethanol or 10⁻³-2 M NaCl. *b*, 56.2% ethanol; the broken line gives the spectrum in 3.9 M NaCl in the absence of ethanol⁸. *c*, 80 w/w % ethanol. *d*, Solid line, 7.1 M CsCl; broken line, 3.8 M LiCl.



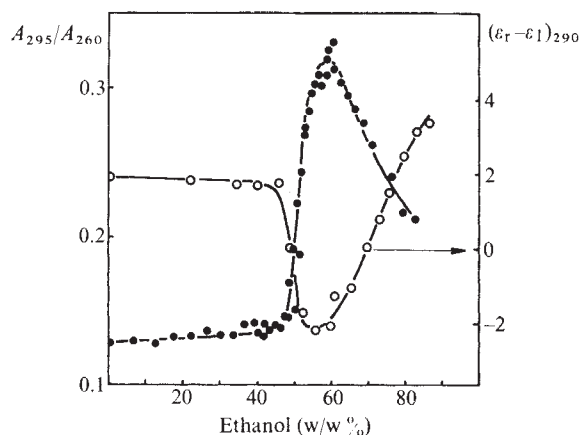


Fig. 2 Optical properties of $(dG-dC)_{100} \cdot (dG-dC)_{100}$ as a function of the ethanol concentration. Titrations were performed by adding 1 mM phosphate buffer or ethanol, respectively. Circular dichroism at 290 nm; absorbance at 295 nm divided by the one at 260 nm.

CsCl a cooperative transition to an inverted spectrum, like the one in Fig. 1b, is observed. At still higher CsCl concentration there is a gradual change to the spectrum shown in Fig. 1d, which is similar to the one obtained in LiCl solutions where no such inversion of the CD spectrum is observed.

The change of the solvent composition alone thus gives four different types of CD spectra for this polynucleotide and it is very likely that each one reflects a different double helical conformation. The type of spectrum in Fig. 1a together with the data on the Raman scattering of poly(dG-dC)¹¹ suggests that in "physiological" conditions the B form is adopted. At very high ethanol concentration the similarity of the CD spectrum (Fig. 1c) with the one for the corresponding RNA⁵ is a strong indication for an A form of DNA, which is observed in fibres or films at very low water activity^{12,13}. On the other hand, the non-conservative spectra at high CsCl or LiCl concentrations (Fig. 1d) closely resembles the one of Li-DNA in films¹³ and may be attributed to the C form.

Assigning a known double-helical conformation to the inverted spectra of poly(dG-dC) in Fig. 1b leaves the D form as the most likely candidate. This hypothesis is supported by the Raman spectrum of this form, which does not show the features characteristic of the A, B or C conformation¹¹. It also offers an explanation for the results of the X-ray diffraction studies with poly(dG-dC) mentioned above. For this type of diffraction pattern models for a right-handed⁴ and a left-handed double helix¹⁴ have been proposed. Although the CD spectrum is suggestive for the second alternative, no clear decision is possible, since calculations show that similar "inverted" CD-spectra can also be obtained for right-handed double helices (unpublished results). Although the assignment of these different double helical forms to the observed CD spectra has to be regarded as tentative, the experiments show nevertheless that relatively small changes of the medium around the DNA, as compared with the binding of proteins, allow a variety of conformational changes to occur in DNA in solution.

At the interface between two different helical conformations within a long DNA molecule one has to postulate for stereochemical reasons 'point defects' of 'dislocations' involving one or more base pairs. Such 'point defects' can also lead to 'kinks' within DNA molecules¹⁵ facilitating the tight packing of DNA chromosomes or viruses.

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Concentration quenching in chlorophyll

IN the primary process of plant photosynthesis it is generally accepted that efficient energy migration occurs between about 300 molecules of chlorophyll *a*, with subsequent light collection by a chemical trap. Solutions of chlorophyll *in vitro*, whether in fluid solvents¹, monolayers^{2,3}, multilayers^{4,5} or, as shown recently in our laboratory, in rigid matrices of PMMA and in bilayer lipid vesicles, exhibit the phenomenon of concentration quenching of the excited state at concentrations much lower than those which are present in the chloroplast. At a chlorophyll concentration of 10^{-1} M, which is comparable with that in the chloroplast, none of the *in vitro* systems has a fluorescent yield as high as is found *in vivo*, especially when the photochemical traps are closed. To try to understand the apparent absence of concentration quenching *in vivo*, we have re-examined its mechanism *in vitro*, and conclude that each chlorophyll molecule in the light-collecting system must be separated from other chlorophyll molecules, so as to prevent trap formation by orbital overlap and so that the minimum distance, averaged over all orientations in a random array, is 10 Å.

A comprehensive review of the earlier work on concentration quenching has been given by Rabinowitch⁶. The fact that quenching occurs equally efficiently in media of high viscosity and in fluid solvents rules out diffusional processes and several authors have recognised that the concentration range of chlorophyll self quenching, in polar solvents such as ether and alcohols, indicates the involvement of Förster-type inductive resonance transfer between chlorophyll molecules^{7,8}. There is no theoretical reason why this, in itself, should result in quenching but it could do so efficiently if a fraction of the molecules present acted as traps. This would be the case when non-fluorescent dimers are present and these undoubtedly account for most of the concentration quenching in such non-polar solvents as hydrocarbons and carbon tetrachloride, where the presence of the dimer is well established and characterised by a new absorption at longer wavelengths. We have made careful studies of absorption spectra in polar solvents such as ethanol and find no evidence of dimer formation at concentrations where extensive quenching occurs, nor are there any concentration-dependent changes in the absorption spectra of the varied systems mentioned earlier. At the highest concentrations used, the dimers would have had to exceed 10% of the total chlorophyll if they were the only traps present.

We therefore propose that the mechanism of concentration quenching in chlorophyll solutions is energy transfer between chlorophyll molecules by inductive resonance, followed by capture at a non-fluorescent trap which, in polar solvents, is merely a pair of chlorophyll molecules the separation of which, although part of the equilibrium statistical distribution, is less than a critical distance R_c . When one of a pair of molecules within this separation is excited, it interacts to form an excimer (fluorescing in the infrared, if at all) or other excited complex, as has been shown to occur in some aromatic hydrocarbons, even in rigid matrices⁹.

To test this mechanism we calculated the rate of a random

walk of the excitation in a random three-dimensional array of molecules by a process in which the intermolecular transfer rate is proportional to R^{-6} (R being the intermolecular separation). The approximations which have to be made to obtain an explicit solution to this problem introduce uncertainties which are difficult to assess and we have therefore used a Monte Carlo method of calculation. By selection of pseudo-random numbers from a uniform distribution, 1,620 molecules are positioned among 27×10^6 coordinates in a regular three-dimensional lattice. This is equivalent to placing points in a cube consisting of 300 planes with separation D Å. Excitation is introduced by selecting one point at random. Rates are then calculated for all the possible processes available to the initially excited molecule, namely, fluorescence, intersystem crossing and resonance energy transfer to all molecules in a sphere of radius $35D$ Å around the excited molecule. The energy is then transferred to a new molecule and the process is repeated until it is terminated by fluorescence, intersystem crossing or trapping by a pair of molecules closer than a distance R_t . The whole random walk was then repeated using further selections of arrays and the results averaged. The complete process was repeated for a range of concentrations, the concentration being changed by varying D . The lattice is large enough for the probability of excitation reaching the edge of the lattice to be negligible.

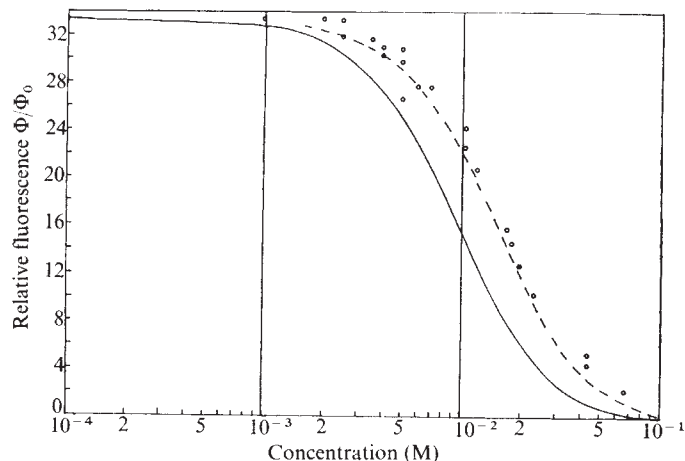


Fig. 1 Relative fluorescence yield of chlorophyll *a* as a function of concentration. ○, Experimental data of Watson and Livingston¹; —, calculated curve with $R_0 = 70$ Å and trap = 14 Å; ---, calculated with $R_0 = 70$ Å and trap = 10 Å.

Complete fluorescence quenching curves for chlorophyll *a*, calculated in this way, are shown in Fig. 1. A separation of the statistical-pair trap was chosen for the calculation of each curve and the process stopped whenever the excitation reached such a pair. In Fig. 1, we have compared our calculations with the classical results of Watson and Livingston¹ for chlorophyll self quenching in ether. The shape of the curve of relative quantum yield against concentration is well reproduced and a quantitative fit is obtained for a statistical-pair trap separation of 10 Å. This may be compared with the diameter of the porphyrin ring in chlorophyll which is 15 Å. In these calculations, the Förster transfer distance R_0 was taken as 70 Å, the fluorescence and intersystem crossing rates as 5×10^7 s⁻¹ and 1×10^8 s⁻¹ which are appropriate for chlorophyll *a*. R_0 was obtained from the overlap of the measured absorption and fluorescence spectra in ethanol using the formulae given by Förster (see ref. 10). Obviously, the average distance of 10 Å between centres in the trap is so small that the orientation will become important. Our purpose here is merely to show how concentration quenching can occur, even in rigid media, when no dimers in the ground state are present. If dimers or oligo-

mers are present, concentration quenching will be expected at even lower concentrations.

We now return to the mechanism of light collection by chlorophyll molecules in the photosynthetic unit. At a chlorophyll concentration in the chloroplast of 10^{-1} M, the fraction of molecules in statistical-pair traps with separation 10 Å would be 0.22 (if they were in random distribution) and efficient photosynthesis would be impossible. Transfer to a chemical trap would have to occur rapidly enough to compete efficiently with trapping by statistical pairs, which is not consistent with the rather high fluorescence yield of the antennae chlorophyll and the correspondingly long fluorescence lifetimes of ~ 100 ps, or even more when the chemical traps are closed¹¹.

Two molecules, which are within the statistical-pair quenching distance, may be prevented from quenching if they are separated by another molecule. Infrared, X-ray diffraction and other structural studies have shown^{12,13} that chlorophyll molecules, whatever their environment, tend to coordinate the Mg atom, either to another chlorophyll or to a solvent molecule, so that chlorophyll with only four bonds coordinated to Mg probably rarely exists. The dimers and oligomers, which Katz suggests¹² comprise the antennae chlorophylls of the chloroplast, are almost non-fluorescent and strongly quenching. We therefore conclude that chlorophyll molecules in the chloroplast antennae, although at close distance, are separated from each other by strongly coordinated molecules which prevent collapse to an excimer or other quenching configuration. It is not yet certain whether antennae chlorophylls are associated with protein, with lipid or with the lipid-protein interface¹⁴. Galactolipids are present in a concentration two or more times that of the chlorophyll¹⁵ and models show that the galactose part of the lipid coordinates well with the Mg of the chlorophyll within the lipid layer, which could thus ensure isolation of each chlorophyll molecule from other chlorophyll molecules. S. E. Carlin (unpublished results), has shown that when lecithin, in lecithin-chlorophyll vesicles, is replaced by galactolipids, the chlorophyll concentration required to give half quenching is approximately doubled, an effect which, although too small to explain the efficiency of the chloroplast, suggests that further work on these lines may be profitable.

Since this work was completed, Fenna and Matthews¹⁶ have reported the first structure determination of a chlorophyll-containing protein, in which they find an irregular arrangement of the rings of the seven chlorophyll molecules, an average nearest-neighbour separation between centres of 12 Å and no evidence of interaction between them. Although this is a bacteriochlorophyll-protein, which is not part of the antennae system, its structure is admirably designed to promote energy transfer while inhibiting concentration quenching.

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Morphological transformation and chromosome aberrations produced by two hair dye components

A POSSIBLE carcinogenic hazard from hair dyes has been suggested recently by mutagenicity studies using the *Salmonella* tester strains developed in Ames' laboratory^{1,2}. We had previously found a good correlation between mutagenicity produced by specific cigarette smoke condensate fractions in the *Salmonella* mutagenesis system³ and oncogenic transformation in the mouse C3H/10T $\frac{1}{2}$ CL8 cell line⁴ developed in Heidelberger's laboratory. I have therefore studied transformation in this cell line after exposure to two hair-dye components, 2-nitro-*p*-phenylenediamine (2-NPPD) and 4-nitro-*o*-phenylenediamine (4-NOPD), both of which had been found to be highly mutagenic in the tester strain TA1538. Although 2-NPPD has greater mutagenic capacity in the presence of a microsomal activation system¹, I thought that 2-NPPD might be able to transform C3H/10T $\frac{1}{2}$ CL8 cells since there is sufficient microsomal activity to convert certain carcinogens such as polycyclic hydrocarbons to their active form(s)⁵. Both 2-NPPD and 4-NOPD were found to produce significant morphological transformation in the C3H/10T $\frac{1}{2}$ CL8 cell line. In addition, in view of the reported correlation between specific chromosome changes and the expression of malignant transformation^{7,8} and the fact that known carcinogens rapidly produce chromosome aberrations⁹, I sought and found chromosome changes after treatment with these hair dye components.

2-NPPD and 4-NOPD, provided by Dr Bruce Ames, were dissolved in dimethyl sulphoxide (DMSO) and incubated at various concentrations with the C3H/10T $\frac{1}{2}$ CL8 for 24 h as described before^{4,6}. Approximately 1 month later the dishes were stained and scored for the presence of transformed foci. Only type III morphologically transformed foci were scored since these produced sarcomas in most cases when isolated and injected into syngeneic C3H mice immunosuppressed by irradiation or rabbit anti-mouse thymocyte serum⁶. The results of two experiments are shown in Table 1. Morphological transformation was found in each experiment after treatment with 2-NPPD in doses

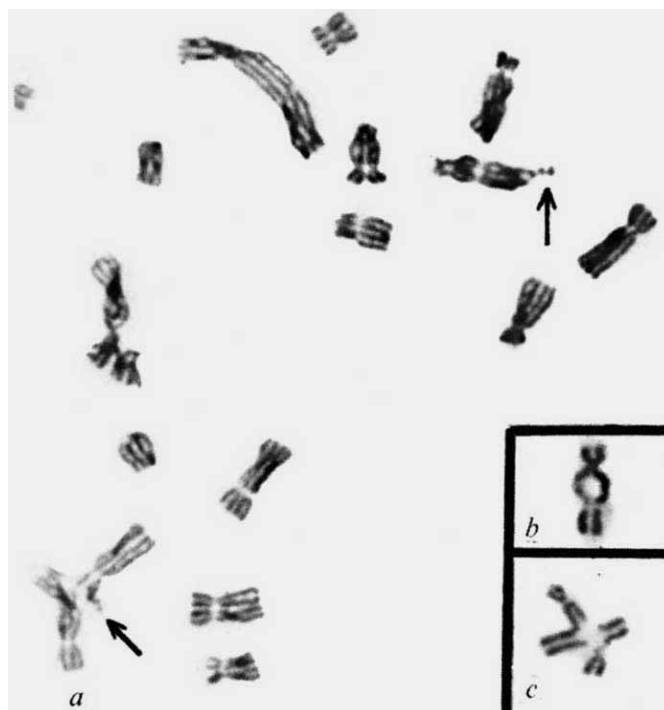


Fig. 1 *a*, Partial metaphase after treatment with 10^{-6} M 2-NPPD showing endoreduplication and two isochromatid fragments (arrows). *b*, An example of a dicentric chromosome seen after 2-NPPD exposure. *c*, A quadriradial chromosome configuration found after treatment with 2-NPPD.

from 10^{-3} M to 10^{-6} M, with an increase in transformation frequency at higher concentrations. Morphological transformation was also produced by 4-NOPD in the same range of doses. A concentration of 10^{-6} M 4-NOPD produced no morphological transformation. Fifteen transformed foci were observed in the dishes treated with 2-NPPD and 4-NOPD, whereas no transformed foci were seen in the control dishes treated only with DMSO. Only one type III focus has been found in DMSO-treated control dishes during the 2.5 yr that the C3H/10T $\frac{1}{2}$ CL8 cells have been used in our laboratory for transformation studies. Transformation was also observed at non-cytotoxic doses of 2-NPPD and 4-NOPD, which further supports the fact that the two hair dye components induce morphological transformation rather than select for it.

The transformation frequency found with these hair dye components was approximately 10 times less than that produced by the potent carcinogenic polycyclic hydrocarbon, 3-methylcholanthrene, at equimolar concentrations⁶. This quantitative comparison, however, is made with caution for several reasons, including the fact that 2-NPPD, 4-NOPD and 3-methylcholanthrene represent different chemical classes and utilise dissimilar activation systems.

The hamster cell line, A(T₁)C1-3, developed in our laboratory was used for the chromosome studies since this cell line contains a diploid number of hamster chromosomes⁸ and is being developed as a rapid screen for chemical carcinogens¹⁰. The results are shown in Table 2. Both 2-NPPD and 4-NOPD produced significant chromatid breaks, with 2-NPPD yielding more chromatid breaks at the highest concentration tested. Several abnormal quadriradial and triradial chromosome formations as well as dicentric chromosomes were seen in metaphases after treatment with 2-NPPD (Fig. 1). The presence of dicentric chromosomes may indicate that 2-NPPD can be active in the G₁ phase of the cell cycle since this type of chromosome abnormality occurs after exposure to a chemical during G₁. Chromatid

Table 1 Morphological transformation produced by 2-NPPD and 4-NOPD

Agent	Dose (M)	Average PE (%)	Total type III foci/total dishes	Transformation frequency (%)
Control (DMSO treated)		15	0/61	<0.01
2-NPPD	10^{-3}	5	3/21	0.29
	10^{-4}	12	2/22	0.08
	10^{-5}	14	1/21	0.03
4-NOPD	10^{-3}	7	2/26	0.07
	10^{-4}	10	2/20	0.10
	10^{-5}	15	5/30	0.11
	10^{-6}	15	0/10	<0.07

Cells were seeded from log-phase mass cultures into 60-mm dishes (1,000 cells per dish) and treated 24 h later with the indicated doses of 2-NPPD or 4-NOPD. Exposure was for 24 h, after which the cultures were washed and maintained for 4-6 weeks. Type III morphologically transformed foci were subsequently scored in stained dishes as previously described^{4,6}. Cytotoxicity was determined in similarly treated cultures containing 200 cells and stained after approximately 10 d. The transformation frequency is the percentage of cells that gave type III transformed foci corrected for the plating efficiency (PE), number of colonies formed as a percentage of number of cells seeded.

Table 2 Chromosome breakage and other chromosomal aberrations produced by 2-NPPD and 4-NOPD

Agent	Dose (M)	No. of metaphases with indicated breaks per cell		Metaphases with indicated chromosome abnormalities
		0	1-4	
Control		50	0	None
2-NPPD	2×10^{-4}	30	20	1 Quadriradial, 3 triradials, 1 Dicentric
	10^{-4}	42	8	1 Quadriradial, 1 triradial, 2 Dicentrics
	10^{-5}	46	4	None
4-NOPD	3×10^{-4}	41	9	None
	10^{-4}	46	4	None
	10^{-5}	47	3	1 Ring chromosome

Hamster cells, A(T₁)C1-3⁸, were exposed for 24 h to various concentrations of 2-NPPD and 4-NOPD. One hour before collection colcemid ($1 \mu\text{g ml}^{-1}$) was added to arrest the cells in metaphase. The chromosome preparations were made as previously described and 50 metaphases for each time period were scored for the presence of chromatid breaks or gaps as well as other chromosome aberrations¹¹. A gap was considered to be a lesion at least the width of a chromatid and a break was a gap with a different angle than the adjacent chromatid arm. All lesions were reported as 'breaks' for simplicity.

breakage is indicative of chromosome damage produced in other phases of the cell cycle.

An endoreduplicated cell was found, after exposure to 10^{-5} M 2-NPPD, which contained two chromosome fragments (Fig. 1). This type of abnormal metaphase may be highly significant in the production of malignant transformation since at this non-toxic dose of 2-NPPD the surviving cell could easily be aneuploid, with chromosome imbalances. We believe such chromosome changes may be related to malignant transformation and oncogenesis^{8,9}. The production of chromosome aberrations by 2-NPPD after exposure of human peripheral lymphocyte cultures has also been noted, although no chromosome abnormalities were found after treatment with 4-NOPD².

Considerable evidence now suggests that certain hair dye components could be potential hazards to humans, especially as these agents can be present in hair dye at concentrations as high as 10^{-2} M (ref. 11). The evidence includes their mutagenic activity in *Salmonella*, their ability to produce morphological transformation in the C3H/10T $\frac{1}{2}$ CL8 cell line and their capacity to yield a significant number of chromosome breaks and rearrangements. Thus further studies of these and other hair dye components for carcinogenic and mutagenic potential *in vivo* seem advisable.

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Mechanism of selective nonspecific cell-mediated cytotoxicity of virus-infected cells

STUDIES of mechanisms of resistance to herpes simplex virus (HSV) in mice have demonstrated antibody-dependent cell-mediated cytotoxicity (AD-CMC) of HSV-infected cells exposed in suspension cultures to anti-HSV sera and normal mouse peritoneal cells (PC) or human peripheral blood leukocytes. In this system it was impossible to demonstrate T-cell-mediated cytotoxicity¹⁻³. In an attempt to adjust the assay conditions to permit detection of such cytotoxicity by immune spleens or PC, the "monolayer" system of Gardner *et al.*⁴ was examined in syngeneic, allogeneic and xenogeneic combinations. Target cell monolayers were infected with HSV and exposed to immune or normal mouse PC. The latter produced unexpectedly high levels of nonspecific release of ⁵¹Cr by the monolayer HSV-infected cells. Because nonspecific or non-selective killing of human tumour cells⁵ by lymphocytes from normal patients has been a major problem in human tumour immunology, we have investigated the mechanism of this nonspecific cell-mediated cytotoxicity in the HSV system. Our results indicate that selective nonspecific killing is a consequence of the cross linking of effector and target cells through aggregated immunoglobulins.

When HSV-infected cells, either Chang human liver cells or neuroblastoma C1300 clone N₂A cells of A strain mice⁶, were exposed to normal uninduced mouse PC, either in "suspension" cultures as described before⁷ or in monolayer cultures in which monolayers were infected for 18 h before addition of the PC, strikingly different patterns of cytotoxicity were observed (Table 1). While only low levels of ⁵¹Cr release were found in suspension cultures of HSV-infected cells, quite high levels of cytotoxicity were found in monolayer cultures of HSV-infected cells and normal PC in a ratio of 1:20. This was a selective non-immune cytotoxicity and not an artefact of the culture conditions, for no such cytotoxicity was produced in identical cultures containing uninfected target cells (Table 1), or target cells infected with measles virus (unpublished observation). Addition of rabbit anti-HSV sera, as reported previously, generated a marked increase of ⁵¹Cr release in suspension cultures attributed to an AD-CMC. In contrast, antibody-inhibited cytotoxicity in the monolayer cultures. The effector cells in this system were almost totally removed by passage over nylon wool columns⁷ and were destroyed by preincubation with silica, known to be cytotoxic for macrophages⁸. Thus the nonspecific cytotoxicity in the monolayer system seems to be related selectively to HSV infection and is probably produced by macrophages.

It is known that infection of various non-lymphoid cells with HSV induces the expression of a receptor for the Fc region of immunoglobulins on the cell surface^{9,10}, and this was confirmed on our target cells. These observations suggested that the selective, nonspecific cytotoxicity observed in the HSV system might be mediated by cross linking of effector cells and target cells, both displaying Fc receptors, by aggregated immunoglobulins or immune complexes. Since most commercial foetal calf sera (FCS) contain some immunoglobulins, the monolayer assay was

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Table 1 Cytotoxicity of peritoneal cells for HSV-infected Chang cells in monolayer or suspension cultures

Cells	Serum	% Specific cytotoxicity*	
		Monolayer	Suspension
PC	—	65±6.3†	5±3.1
PC	Normal rabbit (1/100)	60±6.0	0±3.8
PC	Rabbit anti-HSV (1/100)	41±5.3	89±8.3
—	Rabbit anti-HSV (1/100)	10±1.5	8±1.2
			Uninfected Chang cells Monolayer
			8±1.8
			NT
			12±3.5
			3±1.3

For the monolayer assay Chang human liver cells were dispensed in 1 ml of MEM + 10% FCS into flat bottom wells of multi-dish tissue culture trays (Linbro FB-16-24-TC) at a density of 1×10^5 per well. Three hours later the cells were infected with the HFEM strain of Herpes simplex (HSV) at an input multiplicity of 10. Twenty-four hours later each well was labelled with $2 \mu\text{Ci}$ of $^{51}\text{CrO}_4$ (Amersham) for 1 h at 37°C . Cells were washed three times. Uninfected cultures handled similarly served as controls. The suspension assay was carried out as before¹. Uninfected normal rabbit serum or rabbit anti-HSV (neutralising titre of 1/800), was added to the monolayer or suspension cultures of target cells. PC were collected from C57BL/6 mice and were added at a ratio of 20:1 in a final volume of 1.0 ml. All assays were carried out in triplicate. Spontaneous leakage of ^{51}Cr was 15–20%.

*Percentage specific cytotoxicity was calculated according to the following formula:

$$\frac{\text{mean } ^{51}\text{Cr release in test group} - \text{mean spontaneous } ^{51}\text{Cr release}}{\text{mean } ^{51}\text{Cr release after freeze and thawing} - \text{mean spontaneous } ^{51}\text{Cr release}} \times 100.$$

† + s.e.m. in six to eight experiments.

carried out in medium containing certified agamma-FCS (Microbiological Associates, lot 87839). As Table 2 shows, the nonspecific cytotoxicity of HSV-infected murine neuroblastoma cells was markedly diminished in the agamma-FCS, and specific cytotoxicity could be seen. Introduction of rabbit immunoglobulins aggregated chemically by *bis*-diazotised benzidine¹¹ into the monolayer system significantly augmented the level of cytotoxicity in the agamma-FCS. This rabbit Ig had no detectable neutralising antibody against HSV. In parallel these aggregates blocked the formation of EA rosettes of sheep red blood (SRBC) cells coated with rabbit anti-SRBC antibodies¹² which detect Fc receptors on the effector cells. Nonspecific cytotoxicity-inducing activity of ordinary FCS could be diminished by ultracentrifugation at 100,000g for 1 h and use of the upper third of the serum (Table 2).

We believe that these results may have more general significance than just for the HSV system. Nonspecific cell-mediated cytotoxicity has been a serious problem in the interpretation of microcytotoxicity assays used to assess tumour immunity in man^{3,13,14}. In this regard it is noteworthy that various primary tumours have been shown to exhibit Fc receptors¹⁵. Hersey *et al.* reported nonspecific killing of melanoma lines by activated T lymphocytes possessing Fc receptors¹⁴. Similarly, complement receptor-

bearing cells from blood of patients with infectious mononucleosis indiscriminately killed lymphoblastoid cell lines, whereas purified T cells had specificity only for target cells infected with Epstein-Barr virus¹⁶. Stott *et al.*¹⁷ found that bovine alveolar macrophages were cytotoxic for para-influenza virus-infected targets but uninfected cells, and immune serum blocked this killing—findings similar to ours with the monolayer system. If this model were applicable to human tumour systems, nonspecific or non-selective cytotoxicity *in vitro* might similarly be mediated by linkage of effector and target cells through aggregated Ig or immune complexes. Cross linking through Fc receptors may be one mechanism of selective but nonspecific cytotoxicity, but it is unlikely to be the only one¹⁸.

Although it may be tempting to speculate that such a nonspecific cytotoxicity of cells exhibiting Fc receptors provides some level of non-immune surveillance or resistance to HSV infection or tumour growth, there is no evidence that this mechanism of cytotoxicity *in vitro* can provide protection *in vivo*.

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Table 2 Effect of aggregated Ig and FCS on Fc rosettes and cell-mediated cytotoxicity of HSV-infected neuroblastoma cells in monolayer cultures

Effector system (normal PC plus)	% ^{51}Cr release	% EA rosettes of P C
FCS	45	NT
FCS + anti-HSV serum (1/100)	11	NT
$\alpha\gamma$ -FCS	14	33
$\alpha\gamma$ +FCS+150 mg Agg. Ig	27	9
$\alpha\gamma$ +FCS+15 mg Agg. Ig	19	20
$\alpha\gamma$ +FCS+1.5 mg Agg. Ig	8	30
$\alpha\gamma$ +FCS+0.5% FCS	29	NT
FCS — after ultra- centrifugation (top third)	7	NT
(bottom third)	20	NT

Agg., aggregated

Monolayer ^{51}Cr cytotoxicity tests were carried out as described in Table 1. Target cells were HSV-infected neuroblastoma cells. Ig aggregates (Agg.) were prepared described by Ishizaka *et al.*¹¹ by mixing rabbit Ig (10 mg ml^{-1} ; Cohn fraction II, Pentex) with *bis*-diazotised benzidine (1/5 v/v). Inhibition of EA rosette formation on effector cells was tested as described by Westmoreland *et al.* using rabbit anti-sheep red blood cells antibodies⁶. FCS was ultracentrifuged at 100,000g for 1 h.

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matters arising

Maze-learning ability of *Drosophila melanogaster*

HAY¹ reported that the probability of right-left choices by *Drosophila melanogaster* in a uniformly illuminated multiple-step maze depends on the path through the maze before reaching the choice point. Animals which had initially turned right and were subsequently forced to take five right turns, by blocking the alternatives, had a greater tendency to turn right again than to turn left and vice versa. Hay interprets these findings to demonstrate that *Drosophila* "learn that this sequence of turns enables them to proceed through the maze towards the light" and "that the flies display associative learning. With no apparent positive or negative reinforcement they associate passage through the maze towards the light with a sequence of turns or, more accurately, with a particular orientation towards the light at each choice point, . . .".

In two-step maze experiments (designed for a different purpose) with *D. melanogaster* we observed similar deviations from binomial distribution². These deviations were also found, however, in dark controls and, furthermore, when none of the alternatives in the maze was blocked such that the flies could proceed to the end of the maze by either route. We therefore do not agree with Hay's interpretation but would tend to relate these observations to the correcting behaviour or reverse turning observed from several different species³⁻⁷.

To demonstrate our point of view we have performed several experiments which we report here. Figure 1 shows a scale drawing of the Plexiglas maze⁸ used for these experiments. All dimensions are large compared with the size of *D. melanogaster* except for the inner diameter of the funnels used to provide a one-way passage². The collecting vessels were empty *Drosophila* culture flasks. The experimental

setup was different from that of Murphey⁹ in that our maze included forced turns between first and second choice points. The maze was washed and carefully cleaned before each run. Experiments were performed in complete darkness, at $22 \pm 1^\circ\text{C}$. Of approximately 400 flies (*Drosophila* wild type, Berlin, males plus females, age 3-10 d after eclosion) placed in the starting vessel usually 250 had completed the run after 12 h.

Table 1 shows the results of a typical experiment together with the total of 16 experiments. To eliminate the possibility that an inherent asymmetry of

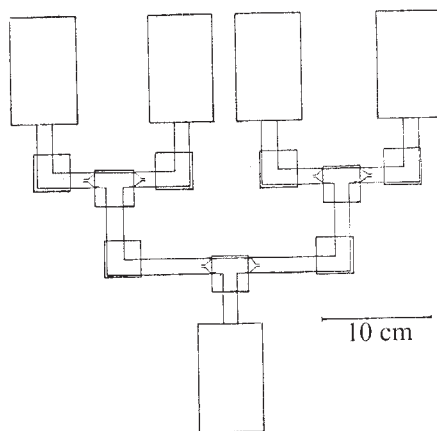


Fig. 1 Plexiglas maze used for experiments with *Drosophila*.

the maze causes the observed distribution, eight experiments were performed in orientation I (*a,b,c,d*) and eight in orientation II (*b,a,d,c*) by permuting the parts of the maze.

Table 1 also gives the average of the choice frequencies and the standard deviation of the mean. P_{LL} is the frequency of two subsequent left choices, P_{LR} is the frequency of a left choice and a subsequent right choice in the maze. It is obvious that $P_{LL} > P_{LR}$ and $P_{RL} < P_{RR}$. The differences are highly significant as tested with a *t*-test and a Wilcoxon rank sign test. The effect

observed in our maze, however, is only about half as large as that reported by Hay¹.

Hay reported an experiment in which all passages in the evenly illuminated maze were allowed and yet a non-binomial distribution of flies was found. For our purposes we have only found it necessary to report that the effect is still observed in the dark. Thus it can hardly be associated with a successful "passage through the maze towards light". It is not intended to give an explanation⁸ of this reverse turning behaviour; however, associative learning seems to be an inappropriate description.

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HAY REPLIES—I welcome the opportunity to expand the arguments in my report¹ on associative learning in *Drosophila*. There I listed and eliminated alternative explanations of the behaviour. Bicker and Spatz² have pointed out that one of these alternatives, namely correcting behaviour, could affect my results. Although I did not use the term 'correcting behaviour', it encompasses two of the variables I considered, namely centrifugal swing and spontaneous alternation.

The crucial experiment, however, (at the top of page 46 in my report), by which I distinguished correcting behaviour from associative learning is impossible in their case as they used only one strain of *Drosophila*. I demonstrated that, although there are strain differences in the probability of following the outer walls of a maze where all choices are available (which is what a correcting behaviour explanation would require), these strain differences are unrelated to the performance in the maze where choices are forced. That is, I accept that correct-

Table 1 Performance of *Drosophila* in maze

	LL	LR	RL	RR
No. of flies counted in collecting vessels in a single experiment	75	45	39	69
Total of 16 experiments	1,331	903	903	1,391
Average of the choice frequencies	$P_{LL} = 0.296$	$P_{LR} = 0.202$	$P_{RL} = 0.196$	$P_{RR} = 0.306$
	± 0.019	± 0.012	± 0.015	± 0.015
In Hay's notation	$P^*_{LL} = 0.602$			$P^*_{RR} = 0.604$
	± 0.021			± 0.021

ing behaviour is involved, but it cannot account for my strain differences in the latter maze in CL and CR (the changes between the first and second choice points in the probabilities of turning left or right) and it is necessary to introduce another variable, namely genetic differences in associative learning.

All Bicker and Spatz have done is to confirm Murphey's³ demonstration that *Drosophila* show spontaneous alternation. This would explain why they obtained similar results in the dark and need not refute my suggestion that the flies associate passage through the maze with visual cues. In any case, one would expect very little learning in their maze, where there is only one forced turn between the two choice points, whereas my maze had a sequence of six forced right-left or left-right turns.

Finally, I should like to stress the need to consider both genetic variation and apparatus differences in maze learning work with *Drosophila*. First, I found large strain differences in learning, and other workers should similarly use a wide variety of strains, rather than just one, if they wish to demonstrate learning. Second, Hay and Crossley (submitted for publication) showed with a maze rather like the one used by Bicker and Spatz but with an extra forced turn, that such apparently minor apparatus variations as the length of the connection between the start-tube and the first choice point can have very large effects on the probabilities of turning left or right. For example, the values of CL for males of the LM 20 strain varied from a mean $\pm$ s.e. of -0.069 ± 0.058 to $+0.491 \pm 0.102$ depending on the apparatus. Thus apparatus specifications need to be considered carefully in replicating the maze-learning work but in turn offer a means of studying in more detail some of the factors that determine turning preferences in *Drosophila*.

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Comments on a closed galaxy model for cosmic-ray propagation

RASMUSSEN and Peters¹ have presented a model of cosmic-ray propagation in which the particles do not escape from the galactic confinement region, but are

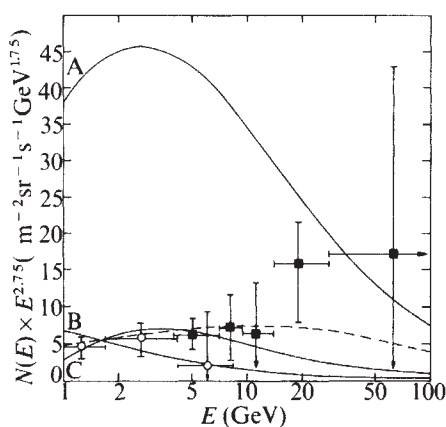


Fig. 1 Positron intensities above 1 GeV. —, Leaky-box model with average matter traversal of 4 g cm^{-2} and $E_{\text{mod}} = 0.4 \text{ GeV}$; —, closed galaxy model with various values of E_{mod} and n_{H} (see text). O, From ref. 3; ■, from ref. 4.

eventually absorbed by meson-producing interactions with the interstellar gas. This contrasts with the generally accepted 'leaky-box' model in which cosmic rays escape from the Galaxy after traversing $\sim 4 \text{ g cm}^{-2}$ of interstellar gas. We consider here the intensities of positrons and γ rays implied by the two models.

In Fig. 1, which is adapted from a figure of Orth and Buffington², the dashed line shows the positron intensity calculated by them for the leaky-box model. For the case of no escape of positrons, appropriate to the closed galaxy model, the solution of the diffusion equation for the positron intensity is

$$N(E) = q_0 E^{-\Gamma} (\Gamma - 1)^{-1} (Eb + a)^{-1}$$

where $q_0 E^{-\Gamma}$ is the rate of production of positrons by the observed equilibrium nuclear flux. Their rate of energy loss is $bE^2 + aE$, the first term corresponding to synchrotron and inverse Compton losses and the second to bremsstrahlung. Both q_0 and a are proportional to n_{H} , the effective mean density of interstellar gas traversed by the cosmic rays (nucleons cm^{-3}). Taking the rate of production of positrons given by Orth and Buffington; and $b = 10^{-16} \text{ GeV}^{-1} \text{ s}^{-1}$ the interstellar positron intensity is

$$N(E) = \frac{514 E^{-2.65} n_{\text{H}}}{(E + 8.0 n_{\text{H}})} \text{ m}^{-2} \text{ sr}^{-1} \text{ s}^{-1} \text{ GeV}^{-1}$$

To compare this with observations, the modulation of the intensity by the interplanetary magnetic field has to be included. The modulation factor is expected to have the form

$$\exp(-E_{\text{mod}}/E) \text{ for } E > 1 \text{ GeV}$$

The leaky-box spectrum in Fig. 1 was obtained using $E_{\text{mod}} = 0.4 \text{ GeV}$ and

$n_{\text{H}} = 1 \text{ cm}^{-3}$. Curve A is our calculated closed galaxy positron spectrum for the same values of these parameters. The observations show that either a lower n_{H} or a higher E_{mod} is required. It is not unreasonable to reduce n_{H} as a larger confinement volume is natural for the closed galaxy model. Curve B is obtained by fixing $E_{\text{mod}} = 0.4 \text{ GeV}$ and varying n_{H} to give a weighted best fit to the observations. The optimum value is $n_{\text{H}} = 0.027 \text{ cm}^{-3}$ implying that the lifetime of cosmic rays in the Galaxy is $\sim 5 \times 10^9 \text{ yr}$. If we regard E_{mod} as a free parameter we obtain the joint optimum values for n_{H} and E_{mod} of 0.1 cm^{-3} and 2.3 GeV respectively (curve C). It is not possible to rule out a modulation as large as this, at least down to 1 GeV , although the non-thermal radio background would favour the smaller value.

Independent evidence is provided, however, by the galactic γ -ray emission above 100 MeV from around the anti-centre direction. Dodds *et al.*⁵ have shown that if nuclear cosmic rays at their local interstellar density filled the disk out to the edge of the Galaxy, their interactions with the observed neutral hydrogen would produce twice as many γ rays as observed. Agreement with observation is obtained if, for instance, the cosmic-ray intensity decreases with galactic radius, R , in proportion to the mass density or if the intensity remains constant out to $R = 12 \text{ kpc}$ and is zero beyond. This type of behaviour is unlikely for the closed galaxy model, which favours a large confinement volume with a uniform cosmic-ray intensity. The γ rays are produced by cosmic-ray nuclei in the 1 – 10 GeV range. $E_{\text{mod}} = 0.4 \text{ GeV}$ was assumed in deriving the local interstellar intensities of nuclei used by Dodds *et al.* from those observed at the Earth. For $E_{\text{mod}} = 2.3 \text{ GeV}$ the intensity could be greater and the discrepancy in the γ -ray flux would be increased by a further factor of two. Thus we conclude that, although the closed galaxy model can be reconciled, as its authors state, with the observed positron flux, there are difficulties in making it agree at the same time with the observed γ -ray flux from the anticentre direction.

We thank A. W. Wolfendale and D. Dodds for helpful discussions.

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reviews

Fish Communities in Tropical Freshwaters: Their Distribution, Ecology and Evolution. By R. H. Lowe-McConnell. Pp. xvii+337. (Longman: London and New York, 1975.) £10.

To those accustomed to the restricted fish fauna of British freshwaters, the diversity in tropical freshwaters is bewildering. More than 8,000 of the 20,000 modern fish species live in tropical rivers and lakes; these include many suborders not represented in higher latitudes, for example, the electric fishes of the unrelated Gymnotoidei and Mormyroidei. The need to refer to many species which will be unfamiliar to most readers poses a problem to the author, who tackles it by providing a general chapter about the fish faunas of Africa, South America and South-east Asia, including five pages of excellent drawings of representative genera, by listing all the families in an Appendix and by giving a separate index of fish names, including synonyms.

Five chapters compare fish communities in the four main types of tropical freshwaters: equatorial forest rivers; seasonal rivers; natural lakes; and manmade lakes. All the lakes are African but Dr Lowe-McConnell is able to compare rivers in the three continents. For each locality, she outlines the topography and geological history, and then describes climate and resulting changes in water conditions. This treatment brings out clearly the similarities between physical and chemical conditions in rivers of the three continents and helps to explain the remarkable convergences in the adaptations of the fishes and the general structure of the communities in these waters. In contrast to temperate waters, there is usually a diurnal and a nocturnal fauna which never meet. The regular flooding of large rivers is associated with the habit of feeding and breeding in the shallow productive flooded areas and then facing the problem of retreating to the restricted river channels when the floods subside.

In three other chapters, Dr Lowe-McConnell makes a valuable contribution to current discussions about the origin and maintenance of diversity in tropical ecosystems by considering topics such as trophic relationships, breeding behaviour and speciation, especially in the African lakes. Her own wide experience in the tropics makes her comments vivid and penetrating.

This book should appeal to a variety of readers: it presents a comparative view of complex fish communities for all those interested in ecology and evolution; it provides a good basis for anyone starting detailed work on tropical fishes, either from an academic or from a practical point of view. The 20-page bibliography will be especially useful to fishery workers since it in-

Tide turns for fisheries research

cludes many papers which are relatively unknown because they are difficult to obtain. The only major complaint to make is the price—at £10, few students are likely to buy their own copies but it is a 'must' for teaching libraries as well as for many research workers. **Margaret Varley**

Fisheries Resources of the Sea and their Management. (Science and Engineering Policy Series.) By David Cushing. Pp. 87. (London: Oxford University, 1975.) £3.50.

THIS book is a brief but useful review of the world's fisheries to inform the uninformed. The first chapter deals with the recent history and development of the fisheries and fishing gear. The world catch of fish amounts to 65–70 million tons, the predominant species being the anchovies, sardines and herrings, followed by the cod-like fishes, together amounting to 25 million tons.

Japan and the USSR have become the biggest fishing nations but fisheries research is practised in most maritime countries, its foundation being based mainly on work in the N.W. European countries. Dr Cushing gives a potted survey of age, growth, fecundity and migrations of fish, and the delimiting of stocks, with a longer chapter on fish population dynamics. Following the earlier regulation of fisheries by mesh sizes of nets, minimum sizes of fish for landing or by close seasons, there has been a trend towards allocating quotas to countries. In spite of research and control,

overfishing is still prevalent. Nevertheless it is considered that up to 100 million tons could be taken annually, mainly by the exploitation of new stocks.

Dr Cushing surveys the history and practice of fisheries management and the various international councils and commissions. The international structure of management is extremely complex and these chapters are especially useful in giving a brief account of the efforts being made to control fisheries in a rational manner. Alas, national interests, fishermen's lobbies and disagreements between governments and scientists have meant that attempts at conservation have often ended in failure.

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Principles of Fishery Science. By W. H. Everhart, A. W. Eipper and W. D. Youngs. Pp. 288. (Comstock: Ithaca, New York and London, September 1975.) £6.85.

Principles of Fishery Science is an updated abridged version of *Fishery Science: Its Methods and Applications* by Rounsefell and Everhart, first published in 1953 and aimed at graduate and upper division undergraduate students. In its original form this book soon became a classic at a time when fishery science was a rapidly expanding field. The authors have endeavoured to update the book in the light of enormous developments in fishery research during the past two decades. They stress, however, that the book is designed to present principles and applications of fishery management, and assumes that students and practising fishery biologists already have a foundation in the biological, physical and mathematical, and social sciences.

The first two chapters made me feel that the authors' endeavours had been successful. The Introduction refers to systems analysis and describes the new approach to research as a result of the general systems theory. The following chapter on Characteristics of Fisheries is also thought-provoking. The subsequent chapter on Fishing Gear, however, seems quite out of place. It interrupts the flow of the book and seems to have been lifted straight out of the 1953 edition.

Chapter 4 on Population Identification sets our train of thought back on the rails and brings us well up to date

with the latest methods for separating populations using serology and X-ray fluorescence spectrometry.

The following five chapters I am afraid contribute nothing new to the field. Many cheaper books (an important consideration for undergraduates), such as those produced for the International Biological Programme, deal with age and growth, population estimation, mortality, recruitment and yield in as much detail.

The authors come into their own when considering habitat improvement, pond management, screens and guiding devices and fish ways, subjects given considerable space in their earlier book. There is a slight imbalance at times (a result of three authors?), for example pollution is "written off" in four pages, although a two and a half

page bibliography on the subject follows the chapter. Yet, under Control of Undesirable Species (Chapter 18), we have a 2-page description (pp263-265) of the new piscicide antimony.

There are two useful chapters on the Role of Hatchery-reared Fish and Stocking, although the literature citations are practically all of work carried out in North America; this is a general criticism of the bibliographies at the end of all the chapters.

This is a useful working text for undergraduates, which I shall certainly suggest mine read. It is difficult, however, to recommend its purchase at its present price. I suggest that its contents be slightly reduced, perhaps by removing chapters 3, 9 and 11, and released as a paperback. It will then serve a very useful role. **Derek Mills**

Surface physics

Surface Physics. (Oxford Physics Series.) By M. Prutton. Pp. viii+117. (Clarendon: Oxford; Oxford University: London, August 1975.) £2.75.

THE 'black magic' of surface effects is just starting to gell into science. Physicists have seen good spectroscopic evidence of the 'dangling bonds' or 'surface states' of which they have talked for years; and now diffraction experiments can identify the reconstruction of the lattice which take place if a layer of atoms is deprived of its neighbours on one side. The concepts, although exciting, are still complex and novel; and thus any attempt to present the physics of surfaces to the undergraduate requires a controlled style, a very firm grip of concepts and will also gain tremendously from deep immersion in the prevailing currents of research. Dr Prutton qualifies in all these departments and has produced a fascinating and readable text, covering a surprising amount in an inexpensive 117 pages. He also somehow manages to combine impartiality with an infectious enthusiasm.

For those solid-state device workers who may be preparing to rush precipitately to the bookshelves, it must quickly be said, however, that the systems covered here are all ultraclean vacuum-solid interfaces and the much more murky field of solid-solid interfaces will probably have to wait a long time before a similar burst of enlightenment manifests itself. Ultrahigh vacuum techniques have made possible a battery of methods (LEED, XPS, UPS, ELS, AES and FEM, to name a few acronymically) which have revolutionised our knowledge of vacuum-solid interfaces but which cannot pene-

trate to internal interfaces. Even the device worker, however, can learn a great amount from this book about the detection of surface impurities and he can also begin to appreciate how the growth of thin films is so powerfully controlled by the surface forces described.

I particularly appreciated some clear explanations of Ewald sphere construction for two-dimensional 'nets' and the distinctions between ultraviolet and X-ray photoelectron spectroscopy. Most of the drawings are jewels of explanatory lore, the captions being nearly sufficient in themselves as a teaching instrument; the references to other texts are confined wherever possible to a few authoritative works.

Among the explanations which I found less than illuminating were those on field emission and the photoelectric effect. Here, surely, are two properties well suited for demonstrating both the ideal electronic behaviour of surfaces and some direct practical uses of clean vacuum-solid interfaces as, for example, in photomultipliers. The great body of work on the effect of alkali atom adsorbates on the electron affinity of surfaces would have fitted rather well into the scheme of the book but is not mentioned. This is, perhaps the most clear example of a tendency in the book to emphasise structural features at the expense of electronic properties; another example is the very brief treatment of the surface state as an electronic level, in spite of some classic studies recently published by Spicer, Eastman and others.

With these reservations there is no doubt that this compact and inexpensive little book will engender the right kind of enthusiasm for and familiarity with surface physics in many universities and even sixth forms.

Andrew Holmes-Siedle

Mexikanischer Vulkane

Studien zur jungquartären Glazialmorphologie mexikanischer Vulkane. (Mexiko-Projekt der deutschen Forschungsgemeinschaft, Vol. VII.) Pp. 178. By Klaus Heine. (Franz Steiner: Wiesbaden, 1975.) Quarto, art. Hardback: DM82.00.

THIS work, based on an extensive field survey of the more accessible high volcanoes, sampling of sediments and mineralogical analyses, is extremely interdisciplinary. The geomorphological features are assigned to their relative sequence and absolute dates through the stratigraphical evidence afforded by intervening beds of pyroclastics and fossil soils. Both of these, being readily identifiable and very widespread in the region and often carrying at least limiting ^{14}C dates, enable correlations to be established between glacial features which are miles distant from each other, on more or less isolated volcanic piles up to 5,500 m in altitude.

At no time, it seems, did even valley glaciers extend below the 2,500 m contour, whereas the three present-day, barely surviving ice caps all lie above 4,700 m. Five main glacier advances, marked by moraines, are recognised: MI, 38,000-32,000; MII, about 12,100; MIII, 1+2, about 10,000-9,000; MIV, c. 2,000; MV, 180-25 yr b.p. These conclusions are supported by numerous sections distributed over a wide area described and figured in detail, and borne out by corresponding evidences on the four volcanoes most closely studied. (La Malinche, Iztaccíhuatl, Popocatepetl, Citlaltépetl.) Similar, but less complete, observations were made on a number of lesser peaks, which, although unglaciated today, show the signs of at least some of the late Pleistocene and Holocene events described.

A brave attempt is made to derive a palaeoclimatic explanation of the ground phenomena, which is, perhaps, less convincing than the results of the painstaking fieldwork. This is largely due to the very divergent views expressed in the climatological literature, so that any author is obliged to espouse one or other mutually exclusive theories.

An enormous Bibliography is provided of relevant literature, making this a valuable work of reference and a worthy successor to previous volumes in the series.

I. W. Cornwall

Hermetic dialogue

The Ash Wednesday Supper: La Cena de le Ceneri. By Giordano Bruno. Pp. 174. (Mouton: The Hague and Paris, 1975.) 28 Dutch guilders.

The Ash Wednesday Supper (La Cena de le Ceneri) is one of six Italian dialogues written by Giordano Bruno for the Elizabethan court when he visited London in 1584–85. The underlying purpose of the dialogue was at once religious and political. Bruno hoped to achieve a union between England and France through a hermetic interpretation of the Christian religion. Historians of sixteenth century intellectual history have found Bruno's work interesting not only for this reason, but because it involves an extended metaphor grounded in Copernican astronomy. Professor Jaki's translation of *La Cena* is based on an edition by Giovanni Aquilecchia of 1955, rather than on the later and fuller edition by the same editor. Professor Jaki makes a very stern critic of Bruno's science in many of its details, and

might have been a more sympathetic commentator had he not insisted on treating Bruno as something he was not, namely a scientist in the modern sense of the word. It will cause no-one surprise to find that Bruno does not conform to the canons of modern scientific thought, and history is not likely to be much illuminated by showing how Bruno failed to do so. Does it matter that Bruno did not anticipate Olbers' paradox? Professor Jaki's translation will certainly make the work of Bruno more accessible to an English-speaking audience. The commentary propagates one or two old myths, such as that of the 50 astronomers engaged by Alfonso of Castile to revise the Ptolemaic astronomical tables. For the most part, the translation reads well, but there are a number of points where the very sense of the English should have drawn attention to a mistranslation. Lewis and Short will soon put right the translation offered for *Neque id sine lepore et gratia*, which can hardly mean "Not without a rabbit and thanks"! **J. D. North**

Porphyrins

Porphyrins and Metalloporphyrins. Edited by K. M. Smith. New Edition based on the original volume by J. E. Falk. Pp. xxiii+910. Elsevier Scientific: Amsterdam, Oxford and New York, 1975.) Dfl. 275; \$114.50.

THIS book will be warmly welcomed by all who work in the area of porphyrins and metalloporphyrins, as it brings up to date material covered by Falk, while changing the emphasis somewhat to cover synthetic aspects and applications of spectroscopic methods. Throughout, the authors have taken care to emphasise biological relevance, and a section dealing with porphyrin biosynthesis and catabolism is included. Apart from a lack of coverage of catalytic properties of metalloporphyrins, the choice of material and its distribution among the various chapters is excellent. The editor is to be further congratulated on the coordination and cross-referencing between various chapters and the absence of significant overlap from different contributors. The presentation is good with many helpful diagrams and formulae, but there is no distinction in the type-setting between different classes of subheadings and, in parts, this is confusing. The text contains an abundance of references to reviews and original papers up to late 1974.

The first section deals with nomenclature and general features of structure, stability and spectra, fol-

lowed by a summary of recent developments in chemical synthesis. Section 2 contains chapters on biosynthesis (including an excellent account of the 'type III isomer problem') and degradation to bilins. A comprehensive account of the coordination chemistry of porphyrins including static and dynamic aspects is followed by a section on methods for determination of molecular structure. This contains a wealth of information on X-ray, mass spectrometric, infrared and Raman studies and, notably, a detailed discussion of nuclear magnetic resonance to porphyrins, metalloporphyrins and related compounds. Applications of electron spin resonance and Mossbauer spectroscopy are considered in subsequent chapters. Later sections deal with chemical reactivity (including developments in photochemistry) and with structural analogues of porphyrins. The book concludes with a detailed and useful section on laboratory methods.

At \$114.50, very few workers in the field will acquire a personal copy and even libraries may prove reluctant purchasers—an unfortunate situation as such a useful book should be within reach of all interested in this field. The problem is only slightly mollified by the news that the Laboratory Methods section is to be published separately. It is to be hoped that a less expensive edition, perhaps in paperback, will soon be forthcoming. **S. B. Brown**

Understanding information processing

Understanding Language: An Information-Processing Analysis of Speech Perception, Reading and Psycholinguistics. Edited by Dominic W. Massaro. Pp. xii+439. (Academic: New York and London, September 1975.) \$16.50; £7.90.

THIS volume comprises eleven chapters; Massaro is an author of six and most of the remainder are by students of his. The book has been edited purposefully so that the coverage and level of the chapters cohere. There are some highly positive general attributes of the book. A wide literature is competently summarised and the references are quite comprehensive as well as up-to-date. To focus a set of reviews of aspects of information processing on the central theme of language is worthwhile.

Massaro's introductory chapter is one of the weaker ones, expounding an overview from a basic flow diagram of the sort which shows how to get from sound waves to abstract memory using only three boxes. Such diagrams are at best a harmless device for distinguishing groups of experiments. Too often they confuse the hierarchical structure of knowledge with the psychological processes by which it is arrived at. This chapter seems to me superficial without being usefully introductory.

The speech perception section begins with a much needed chapter on basic articulatory and acoustic dimensions which facilitates access to a literature which usually takes these as read. Two chapters on acoustic features and temporal factors lead to a review of major theories. Three chapters on reading cover elementary feature processing, word recognition and eye movements. Too much space is devoted to the scholastic enterprise of compiling letter feature lists but the discussion of redundancy utilisation is thorough. Massaro denies the use of lexical redundancy, probably incorrectly. The closing section on psycholinguistics reviews matters adequately treated elsewhere but adds completeness to the volume.

This book offers few significant theoretical insights but it provides a very competent review of an important and mobile area of research and will be highly useful to the advanced undergraduate or specialist for two or three years.

Leslie Henderson

Pure gold

Lysosomes in Biology and Pathology, Volume 4. (North-Holland Research Monographs: Frontiers of Biology, Volume 43.) Edited by J. T. Dingle and R. T. Dean. Pp. xviii+614. (North-Holland: Amsterdam and Oxford; American Elsevier: New York, 1975.) \$74.95; Dfl.180.00.

THE 'gold rush' following the discovery of lysosomes has now subsided. The easily accessible nuggets have been removed and it is now necessary to dig more deeply and systematically to reap rewards. That they are still there is illustrated by several chapters in this volume, which report thorough analyses of old problems, such as the role of lysosomes in degradation of plasma proteins (G. Gregoriadis) and in intracellular protein turnover (H. L. Segal). Several chapters are devoted to lysosomes in different cell types (skeletal and cardiac muscle cells, blood platelets, arterial walls, skin, *Tetrahymena*) but these accounts are more than descriptive, embracing the participation of lyso-

somes in the physiology and pathology of the cells concerned. For example, blood platelets are rich in lysosomes, and the release of their enzymes contributes to the pathogenesis of inflammation; and storage of lipids in lysosomes of cells in arterial walls may be important in atherosclerosis.

Evidence is presented of heterogeneity of lysosomes within single cell types (M. Davies) and of multiple forms of lysosomal enzymes (R. T. Dean). Speculative chapters include those of C. M. Szego on lysosomes in nucleocytoplasmic communication and L. J. Ignarro on the regulation of lysosomal enzyme secretion. In these days when relevance is so much emphasised it is pleasing to see a beautifully illustrated account by Yolande Heslop-Harrison of enzyme release in carnivorous plants. As in previous volumes, the production is of high quality, with many illustrations, including well-reproduced half tones, and comprehensive author and subject indices. The quality of production is unfortunately reflected in the price.

A. C. Allison

Starch structure

Starch and its Components. By W. Banks and C. T. Greenwood. Pp. xi+342. (Edinburgh University: Edinburgh, 1975.) £10.00.

STARCH, particularly as it occurs in cereal grains, is the major carbohydrate food ingredient in the human diet. Yet the structure of the starch granule is still unknown and no one has synthesised a granule *in vitro*. Nevertheless a great amount is known about starch granules and particularly about the structure, behaviour and synthesis of its major molecular components, amylose and amylopectin.

This book reviews the chemical nature of starch and its components, and their behaviour with enzymes. The book is a highly biased presentation and as the authors state in the Preface "We make no apology for the fact that emphasis in this monograph is on our own work . . ." Not everyone will agree with all the authors' statements in the book, but I find the total coverage good and the many arguments and criticisms sound. A reader will carry away a fine sense of starch behaviour and a good feel for the structure and reactions of amylose and amylopectin. Emphasis is placed on hydrodynamic behaviour and on the action of enzymes on amylose and amylopectin.

After a short introduction the book is separated into five major sections. The first deals with the fractionation of starch and the fine structure of its

components. Several fractionation procedures are discussed and the chemical but mainly enzyme reactions of components are given. Emphasis is placed on the use of enzymes to elucidate starch molecular structure. A section of 43 pages is devoted to the reaction of starch and its components with iodine. A section concerned with the conformation of amylose in solution provides a wide coverage of methods for conformational determination and possible interpretations. It is agreed that the basic helical nature of amylose predominates in its complexes and is lowest, if existent at all, in neutral aqueous solution. Starch-degrading enzymes are treated by examining the action pattern of exo-, endo- and branching or debranching enzymes. Pitfalls can be numerous in the use of enzymes to deduce starch structure and many of these are clearly indicated. The final major section carries a discussion of the structure and biosynthesis of the starch granule. Attention is directed toward crystalline nature and orientation.

This is not a practical book. It does not discuss the characteristics of starch pastes nor of the preparation or use of starch or its modifications. The book is devoted to a discussion of the basic structure of starch and its components as interpreted by the authors. As such it is recommended to all those having an interest in the fundamental characteristics of starch. Biochemists will find the review useful and interesting.

Roy L. Whistler

Amateur astronomers' Bible

Astronomy: A Handbook. Edited by G. D. Roth. Translated and Revised by Arthur Beer. Pp. xviii+567. (Springer: Berlin and New York, 1975.) DM44.90; \$18.50.

THIS is a revised and translated version of what was for long the German amateur astronomers' bible, *Handbüch für Sternfreunde*, described rather misleadingly on the dust jacket as "designed to fill the needs of both professional and amateur astronomers". It in fact sets out to encourage the amateur to make and reduce observations of professional quality and rigour, so that the two might be complementary.

The ground covered is naturally similar to that in other amateurs' handbooks such as J. B. Sidgwick's *Observational Astronomy for Amateurs* and *Amateur Astronomer's Handbook*, but its approach is more sophisticated. The chapter on 'Applied Mathematics for Amateur Astronomers' requires maths of at least A-level standard, and several expressions elsewhere in the book would be clarified by the use of worked examples. Yet the handbook is curiously out of date in parts; the radio astronomy chapter contains circuit diagrams which abound in valves, and some of the definitions in this and other chapters have a ring of history about them. There are some strange omissions too, in what is overall a very comprehensive text. Narrow band and even white light observations of solar flares are not discussed; meteor triangulation is dismissed in one sentence, and meteor spectroscopy, where the amateur can contribute important results, is not even mentioned.

There are, however, some particularly good chapters which are not covered well elsewhere. 'Fundamentals of Spherical Astronomy' is an excellent compact reference to position and time measurements. The chapters on solar eclipses, lunar eclipses and artificial Earth satellites are also most informative, and that on 'Observation of the Planets' stresses the amateur's role in continuously monitoring changes in planetary detail. There is a useful and complete appendix; and although several of the original references in the text are necessarily in German, the very thorough bibliography suggests much in the way of alternative reading.

Although this book is unlikely to attain the popular appeal of Sidgwick's handbooks, it is a good reference work for the advanced amateur astronomer and will be welcomed by many.

Heather A. Couper

obituary

Rudolph Minkowski, an outstanding contributor to many areas of astrophysics, died on January 4, 1976, at the age of 80. As a staff astronomer at the Hale Observatories, and later at the Berkeley Radio Astronomy Laboratory, he pioneered the study of planetary nebulae, supernovae, and radio-emitting galaxies.

Minkowski was born in Strassburg and educated at Breslau. Although strongly interested in astronomy, he found the physics program more attractive at Breslau, and he took his Ph.D. in optics in 1921. After a year at Göttingen, he joined the physics group at Hamburg, where he rose to the rank of professor. In 1935 the increasing Nazi repression drove Minkowski to leave his homeland. Encouraged by former colleague Walter Baade, who had preceded him to Pasadena, he moved to a research assistantship at the Mount Wilson Observatory, where his abilities soon earned him a regular staff position. In his 25 years with the telescopes of Mount Wilson, and later of Palomar Observatory, Minkowski became one of the World's leading investigators of the violent phenomena of the universe.

Supernovae remained one of Minkowski's central interests during his entire

career. He distinguished early between the two principal types of supernovae and studied the spectra of many individual supernovae in other galaxies. In collaboration with Baade he studied the remnants of the few known supernovae that have appeared in our own Galaxy. Of particular importance was their thorough analysis of the Crab Nebula, an object whose importance in astrophysics has increased as it was discovered successively to be a radio source, an X-ray source, and a pulsar.

After the discovery of discrete radio sources, Minkowski was a leader in their identification and interpretation. Again working with Baade, he identified some of the strongest radio sources as supernova remnants. Others were identified as disturbed galaxies; the original Baade-Minkowski interpretation of them as pairs in collision did not hold up, but their nature has continued to puzzle astrophysicists for two decades.

Another of Minkowski's long-term interests was the nature of planetary nebulae. In addition to his analysis of these objects, he set up a survey, using a 10" telescope, that more than doubled the number of planetary nebulae known.

Minkowski's greatest public service in astronomy, however, was his supervision of the National Geographic

Society-Palomar Observatory Sky Survey. This incomparably valuable set of photographs has been made widely available in reproduction and is an essential part of the facilities of every astronomical library. Its uniformly high quality is a result of Minkowski's painstaking care.

The study of supernovae and disturbed galaxies naturally led Minkowski to study normal galaxies as well. His pioneering study of internal motions in elliptical galaxies was superseded only a dozen years later, when a new generation of observing equipment became available. It was in his last observing run at the Palomar 200" telescope that he determined the optical redshift of the radio source 3C 295, which remained the farthest point on the velocity-distance diagram of cosmology for 15 years.

After retirement from Mount Wilson and Palomar Observatories in 1960, Minkowski spent a year at the University of Wisconsin and then moved to Berkeley, where he retired again in 1965. Neither retirement had any visible effect on his output of scientific articles, which continued into the 1970s. Several generations of astronomers will remember the personal warmth that underlay his wisdom.

Ivan R. King

announcements

Awards

The Chemical Society, Washington, has awarded the 1975 Hillebrand prize to **Dr Ming-Chang Lin** for his work on and with chemical lasers.

Dr Harold A. Rosen has been awarded the first L. M. Ericsson International Prize for his work on geostationary communications satellites.

The Institute of Physics has made the following awards for 1976:

Charles Vernon Boys Prize to **Professor S. D. Smith** of Heriot-Watt University, Edinburgh, for his contributions to the design of scientific instruments in solid state physics and in physical meteorology.

Duddell Medal and Prize to **Mr G. N. Hounsfield** of EMI Limited, for his development in the use of X rays for

the examination of three dimensional structures.

Glazebrook Medal and Prize to **Sir Montague Finniston** of the British Steel Corporation, for his leadership in the application of science to the large scale manufacture of steel.

Guthrie Medal and Prize to **Professor A. Salam** of the Imperial College, London, and the International Centre for Theoretical Physics, Trieste, for his contributions to the theory of fundamental particles.

Maxwell Medal and Prize to **Dr S. W. Hawking** of the University of Cambridge, for his contributions to theoretical astrophysics.

Rutherford Medal and Prize jointly to **Professor R. J. Blin-Stoyle** of the University of Sussex, and **Dr Joan M. Freeman** of the UKAEA, Harwell, for their work on β -radioactivity of complex nuclei.

Appointments

Sir Kenneth Berrill, **Professor C. C. Booth**, **Sir Alan Cottrell** and **Mrs J. E. Floud** have been appointed to the Advisory Board for the Research Councils (ABRC).

Dr J. H. Humphrey, Deputy director of the National Institute of Medical Research, has been appointed Professor of Immunology at the Royal Postgraduate Medical School.

International meetings

April 1-2, **The Changing Environmental Conditions in Great Britain and Ireland** during the Devensian Cold Stage, London (The Executive Secretary, The Royal Society, 6 Carlton House Terrace, London SW1Y 5AG). April 20-23, **Human Reflexes and Motor Disorders**, Brussels (I.C.B.,

Chaussée de Waterloo, 715, B-1180 Brussels, Belgium).

May 14-15, **Platelets and Thrombosis**, Milan (Dr D. C. B. Mills, Specialised Centre on Thrombosis Research, Temple University, Health Sciences Centre, 3400 N. Broad St., Philadelphia, Pennsylvania 19140).

June 14-19, **4th International Conference on Marine Corrosion and Fouling**, Juan-les-Pins, France (Congress Secretariat, CREO-73, 77, rue de Sèvres, 92100 Boulogne-Sur-Seine, France).

July 13-17, **Biology and Taxonomy of the Solanaceae**, Birmingham (Mr David Langley, Conference Secretary, Department of Botany, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK).

July 26-28, **2nd European Colloquium on Hypothalamic Hormones**, Tübingen (deadline: April 15) (Professor Wolfgang Voelter, Chemisches Institut, Auf der Morgenstelle, 7400 Tübingen, FRG).

January 17-19, 1977, **The Geology, Mining and Extractive Processing of Uranium with Special Reference to Europe**, London (Deadline for abstracts: May 1) (The Secretary, Institution of Mining and Metallurgy, 44 Portland Place, London W1N 4BR, UK).

Reports and publications

United States Department of the Interior: Geological Survey. Water-Supply Paper 2030: Summary of Floods in the United States During 1969. By J. K. Reid. Pp. viii + 173. Water-Supply Paper 2153: Quality of Surface Waters of the United States, 1970. Part 3: Ohio River Basin. Pp. x + 444. (Washington, D-C: S Government Printing Office, 1975.) [1212]

Royal Ontario Museum. A Selected Bibliography on Mercury in the Environment, with Subject Listing. By Susan Robinson and W. B. Scott. Pp. 54. (Life Sciences Miscellaneous Publication.) \$1.25. Life Sciences Contributions. No. 99: A New Genus of Freshwater Triclad from Tasmania, with Reviews of the Related Genera *Cura* and *Neppia* (Turbellaria, Tricladida). By Ian R. Ball. Pp. 48. \$2. No. 100: A Revision of the Latipinnate Ichthyosaurs of the Lower Jurassic of England (Reptilia: Ichthyosauria). By C. McGowan. Pp. 30. \$1.50. No. 101: Redescription of Type Specimens of the Bryozoan *Heterotrypa* from Upper Ordovician Rocks of the Credit River Valley, Ontario, Canada. By Madeleine A. Fritz. Pp. 30. \$1.75. No. 102: Fauna and Correlation of the Ravenscrag Formation (Paleocene) of Southwestern Saskatchewan. By L. S. Russell. Pp. 53. \$2. No. 103: Tertiary Mammals of Saskatchewan. Part 3: The Miocene Fauna. By John E. Storer. Pp. 124. \$4. No. 104: Observations on the Biology of Some Rhodesian Bats, Including a Key to the Chiroptera of Rhodesia. By M. B. Fenton. Pp. 27. \$1.25. No. 105: The Morphology, Karyology and Taxonomy of a New Freshwater Planarian of the Genus *Phagocata* from California (Platyhelminthes: Turbellaria). By Ian R. Ball and N. Goubault. Pp. 19. \$1.50. Occasional Papers. No. 26: Thermal Relations of the Neotropical Frog *Hyla labialis* (Anura: Hylidae). By D. Valdivieso and J. R. Tamsitt. Pp. 12. No. 27: Chromosomes of Fifteen Species of Bats (Chiroptera) from Kenya and Rhodesia. By R. K. Paterson and David W. Nagorsen. Pp. 16. No. 28: Karyotypes of Six Species of Bats (Chiroptera) from the Dominican Republic. By David W. Nagorsen and R. L. Paterson. Pp. 8. (Toronto: Royal Ontario Museum, 1974 and 1975.) [1512]

Peabody Museum of Natural History, Yale University. Bulletin 40: On the Biology of Cosmine. By Keith Stewart Thomson. Pp. 59. (New Haven, Conn: Peabody Museum of Natural History, 1974.) [1512]

Norsk Polarinstittutt. Skrifter Nr. 162: The Ordovician Trilobites of Spitsbergen. II. Asaphidae, Nileidae, Raphiophoridae and Telephindidae of the Valhallfonna Formation. By R. A. Fortey. Pp. 207 (41 plates). (Oslo: Norsk Polarinstittutt, 1975.) [1512]

Australia: Commonwealth Scientific and Industrial Research Organization. Division of Soils Technical Paper No. 25: Cultivation and Fertilizer Effects on the Growth and Foliage Nutrient Concentrations of *P. radiata*, *P. pinaster* and *P. caribaea* on Three Soil Types at Anglesea (Victoria). By M. Raupach, A. R. P. Clarke, B. F. Gibson and K. M. Cellier. Pp. 20. (Glen Osmond, SA: CSIRO, Division of Soils, 1975.) [1512]

Environment Canada: Fisheries and Marine Service.

Person to Person

Scientist offers exchange of comfortable 3-bedroom house in Cambridge for a similar one in Paris from May 15 to July 30. (Contact Dr M. Levitt, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK).

Six months' notice is given of the possible use of plenary powers by the International Commission on Zoological Nomenclature in connection with the following names (see *Bull. zool. Nom.*, 32 (1976)):

- 260 *Dermacentor andersoni* Stiles, 1908 (Acarina: IXODIDAE): proposed conservation.
- 1772 *Ophiura* Lamarck, 1801 and *Ophioderma* Müller and Troschel, 1840: revised proposals for stabilisation.
- 2097 *Ophiopsis* Müller and Troschel, 1840: proposed designation of type-species.
- 2112 CIRCINAE in Aves and Mollusca: proposals to end the homonymy.
- 2113 *Pterois zebra* Quoy and Gaimard, 1825 (Pisces: SCORPAENIDAE): proposed suppression.
- 2116 *Halecium* Oken, 1815 (Coelenterata: Hydroida): Proposed validation: *Thoa* Lamouroux, 1816, proposed suppression.
- 2119 *Lecanium acuminatum* Signoret, 1873 (Hemiptera: COCCIDAE) proposed designation of neotype.
- 2120 *Cystioceras* Börner, 1912 (Insecta: Collembola): proposed suppression.

Comments within 6 months to the Secretary, International Commission on Zoological Nomenclature, c/o British Museum (Natural History), Cromwell Road, London SW7 5BD, UK.

There will be no charge for this service. Send items (not more than 60 words) to Martin Goldman at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

Technical Reports. No. 581: Identification, Host Sites and Biology of Parasites Infecting Juvenile Atlantic Salmon (*Salmo salar*) in the Miramichi River System. By G. M. Hare and M. D. B. Burt. Pp. 34. No. 582: YRAREA—a Program to Demonstrate Effects of Exploitation on a Contagiously Distributed Shellfish Population. By L. E. Gales and J. F. Caddy. Pp. 79. (St. Andrews, NB: Research and Development Directorate, Biological Station, 1975.) [1512]

Geological Survey of Canada. The Geology of Long Beach Segment, Pacific Rim National Park and Its Approaches. By A. H. Lang and J. E. Muller. Pp. 56. \$2.40. Bulletin 249: Upper Cretaceous Stratigraphy, Yukon Coastal Plain and Northwestern Mackenzie Delta. By F. G. Young. Pp. xii + 83. \$7.20. Paper 74-29: Bylot Island Map-Area, District of Franklin. By G. D. Jackson and A. Davidson. iii + 12. \$2.40. Paper 74-48: Major Rock Units of the Morin Complex, Southwestern Quebec. By R. F. Emslie. Pp. iv + 37. \$4.20. Paper 95-1, Part C: Report of Activities, Part C. Pp. vi + 371. \$6. Paper 75-5: Current Research in the Geological Sciences in Canada, 1974/75. Compiled by Thomas E. Bolton. Pp. 107. \$2.40. Paper 75-32: Hesqui Formation (New)—Neritic Channel and

Interchannel Deposit of Oligocene Age, Western Vancouver Island, British Columbia. By J. A. Jeletzky. Pp. 95 (6 plates). \$4.20. (Ottawa: Information Canada, 1975.) [1512]

United States Department of the Interior: Geological Survey. Professional Paper 437-D: Land Subsidence Due to Ground-Water Withdrawal, Arvin-Maricopa Area, California. By Ben E. Loggren. Pp. iv + 55. Professional Paper 827: Geology of the Sierra Foothills Melange and Adjacent Areas, Amador County, California. By Wendell A. Duffield and Robert V. Sharp. Pp. iii + 30 + 1 plate. Professional Paper 857: Diagenesis and Stratigraphy of the Lisburne Group Limestones of the Sadlerochit Mountains and Adjacent Areas, Northeastern Alaska. By George V. Wood and Augustus K. Armstrong. Pp. vi + 47 + 12 plates. \$2.25. (Washington, DC: US Government Printing Office, 1975.) [1512]

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National Hydatids Council. 15th Annual Report and Statement of Accounts for the year ended 31 March 1975. Pp. 24. (Wellington, New Zealand: National Hydatids Council, Ministry of Agriculture and Fisheries, 1975.) [1612]

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